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## THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY

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# THE POTASSIUM EFFLUX AND INFLUX IN YEAST AT DIFFERENT POTASSIUM CONCENTRATIONS [1]

A. ROTHSTEIN AND M. BRUCE

*From the Division of Pharmacology, Department of Radiation Biology, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.*

SIX FIGURES

The uptake of potassium by yeast during the metabolism of exogenous substrate, first demonstrated by Pulver and Verzar('40), has been characterized as an exchange of extracellular potassium for hydrogen ion derived from metabolism (Rothstein and Enns, '46; Conway and O'Malley, '46). Leakage of potassium out of the cell has also been observed. For example, Hevesy and Nielsen ('41) found that there was a greater uptake of $K^{42}$ than of chemical potassium, indicating an exchange of cellular $K^{39}$ for $K^{42}$, in addition to the net uptake of potassium. A slow leakage of potassium ion from the cell, largely in exchange for hydrogen ion, occurs during periods of starvation (no substrate) (Rothstein and Enns, '46; Scott et al., '51).

Although the net movements of potassium into the yeast cell have been characterized in some detail (see reviews of Rothstein, '55, and of Conway, '54), the separate contributions of efflux and influx to the net effect have not been adequately assessed, nor have experiments been carried out at low potassium concentrations. Such studies have been handicapped by technical problems. Firstly, the cells rapidly alter the potassium concentration of the medium especially at low concen-

[1] This paper is based on work performed under contract with the U.S. Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester, N. Y.

For K-free media, double distilled water was passed through an ion-exchange column. The pH was adjusted with HCl.[3] In most experiments no buffer was used, because the flow of medium through the cell-column was rapid enough to minimize pH changes associated with metabolism in the pH range of 2 to 5. Buffer cations are avoided because they increase the rate of K-efflux particularly at pH levels above 4.5.

$K^{42}$ was obtained from the AEC, Oak Ridge, Tenn. It was counted in metal planchets with a thin window Geiger tube.

Yeast concentrations were determined turbidometrically in a colorimeter after calibration against hematocrit volumes.[4]

TABLE 1

*Flow rates through columns of yeast cells at a pressure of 8 lbs./in.*[2]

| AMOUNT OF YEAST | FLOW RATE |
|---|---|
| *mg* | *ml/min.* |
| 400 | 7.2 |
| 600 | 7.0 |
| 800 | 4.5 |
| 1000 | 4.0 |
| 1200 | 3.6 |
| 1400 | 3.2 |
| 1600 | 2.7 |

## RESULTS

Yeast cells given a substrate such as glucose rapidly absorb potassium from the medium. From figure 1, it can be seen that the rate of K-uptake and the maximal amount of uptake are conditioned by the potassium concentration in a non-linear fashion. An increased concentration in the ratio of 1 : 10 : 100 induces an increased rate in the ratio of 1 : 5 : 10. The uptake ceases after a short time, even though there is still ample

[3] The pH determination must be on a separate aliquot, because the calomel electrode leaks traces of potassium.

[4] In packed yeast, after centrifugation, volume of distribution studies with insulin, peptone and gelatin indicate that 22 to 24% of the volume is occupied by trapped medium as compared to the theoretical value of 26% for close packed spheres (Conway and Downey, '50). These results were confirmed in the present study and a correction factor of 22% was applied to the hematocrit determination of cell volumes.

substrate as well as relatively large amounts of potassium left in the medium (only 20% of the potassium is absorbed in the upper two curves, and 50% in the lower curve). In fact there is some return of potassium to the medium at the higher concentrations.

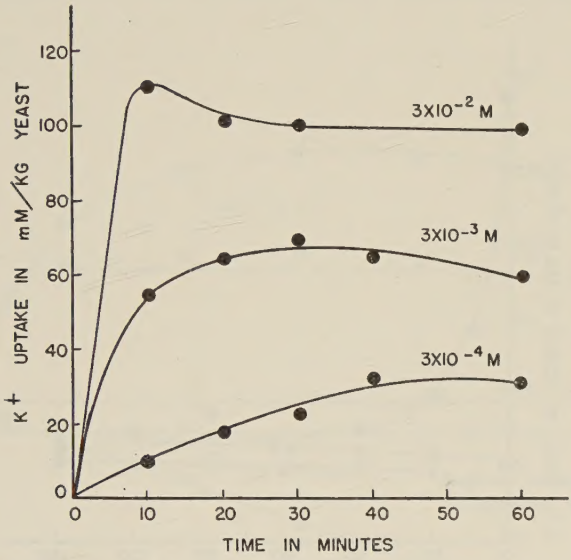


Fig. 1 The uptake of potassium by fermenting yeast at different concentrations of potassium.

The yeast concentrations were 5, 10 and 40 mg/ml of suspension for $3 \times 10^{-4}$, and $3 \times 10^{-3}$, and $3 \times 10^{-2}$ M $K^+$. The glucose concentration was 0.1 M and pH 4.5, with TST buffer.

In another experiment, lower concentrations of potassium as well as K-free medium were used (figure 2). The uptake and loss of potassium from the cells are, in this case, expressed in terms of alterations of the K-concentration of the medium. With $3 \times 10^{-4}$ M K, there was a rapid uptake which ceased after the concentration in the medium was reduced to $5 \times 10^{-5}$ M. With K-free medium, potassium was lost from the cells, but the loss ceased when the concentration of potassium in the medium reached a level of $5 \times 10^{-5}$ M. If the initial concentration was $5 \times 10^{-5}$ M, no changes were observed. Ap-

parently, when the concentration of potassium in the medium is $5 \times 10^{-5}$ M, a steady state obtains in which the rate of uptake and leakage are exactly equal.

The influence of several factors on the steady state were investigated. No marked differences were found between fermenting and respiring yeast. There was little change in the

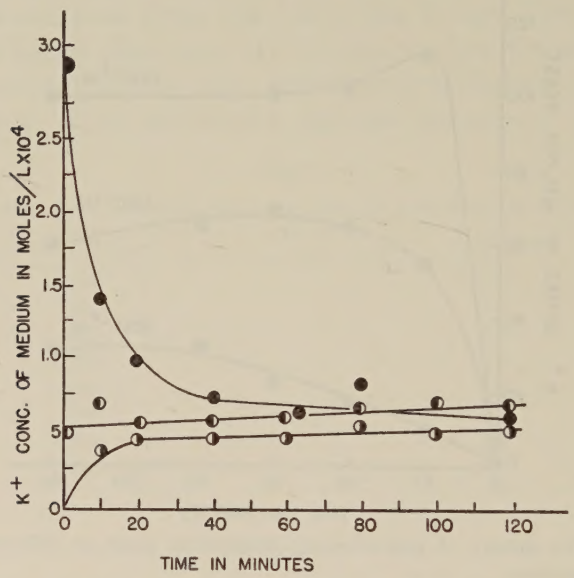


Fig. 2 Change in the potassium concentration of the medium in the presence of fermenting yeast.

The yeast concentration was 10 mg/ml, glucose 0.1 M, pH 4.5 with TST buffer.

steady state if the temperature was raised from 15° to 25°C, but at 35°C it was twice as high as at 25°C. The most dramatic changes were produced by changes in pH. For example, at pH 3.5, the steady-state value was $5 \times 10^{-4}$ M compared to $5 \times 10^{-5}$ M at pH 4.5. The pH dependence is a reflection of the absorption of potassium in an exchange reaction for $H^+$ (Rothstein and Enns, '46; Conway and O'Malley, '46).

The steady-state situation can be disturbed if after a given period of time the cells are separated from the medium by centrifugation and resuspended in fresh medium containing

the original concentration of potassium. For example, in figure 3, data are presented for an experiment in which the cells were resuspended in fresh medium at frequent intervals.

For comparison, data are also presented for an experiment in which the medium was not changed (taken from data of fig. 2). With K-free medium, each time the medium was

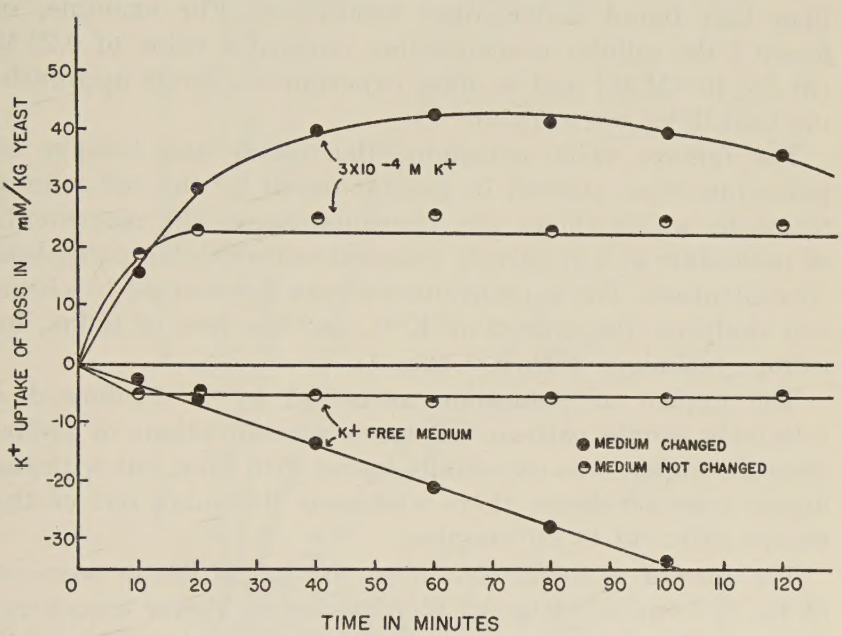


Fig. 3 The uptake and loss of potassium by fermenting yeast when the medium is frequently replaced.

The yeast concentration was 10 mg/ml, glucose 0.1 M, pH 4.5 with TST buffer.

changed another increment of K was lost by the cells so that the total loss of potassium increased continuously with time. In the presence of potassium (initial concentration, $3 \times 10^{-4}$M) the behavior was quite different. The total quantity of potassium absorbed was greater when the medium was changed. However, the increment absorbed was smaller on each successive renewal of medium. In fact, after 60 minutes there was a loss rather than a gain of potassium,

despite the exposure of the cells to the same concentration as initially. The termination of K-uptake after 60 minutes was not due to a decreased metabolism, for the cells were respiring as rapidly the second hour as the first. Furthermore, the cellular concentration of potassium had not reached a limiting maximum value, having risen from an initial value of 0.12 M/kg of cells to only 0.16 M/kg, considerably less than that found under other conditions. For example, in figure 1 the cellular concentration reached a value of 0.22 M (at $3\times 10^{-2}$M K) and in other experiments, levels approaching 0.30 M/kg were found.

The factors which determine the uptake and leakage of potassium were studied in greater detail by the cell-column technique which allows the determination of the movements of potassium at a relatively constant extracellular potassium concentration. The net movements were determined by chemical analyses (designated as $K^{39}$), and the rate of influx, by isotope technique with $K^{42}$ (fig. 4).

The uptake of potassium measured by $K^{42}$ followed a relatively simple pattern. At lower concentrations of potassium the uptake was essentially linear with time, but with the higher concentrations, there was some flattening out of the curves after 40 to 60 minutes.

The rate of K-influx (based on the initial linear portions of the $K^{42}$ curves of fig. 4) increased with higher concentrations of potassium in an asymptotic relationship which could be fitted by the Michael-Menten relationship,

$$1/R = Km/CVm + 1/Vm$$

where R = rate, C = Concentration, Vm = maximal rate and Km = the Michaelis constant. In figure 5, 1/R is plotted against 1/C. The 6 points obtained from figure 4 are plotted (solid line). The intercept in this case was too close to the origin for a graphic determination, so the 4 highest concentrations were replotted on a scale larger by a 10-fold factor (dotted line). The Vm can be roughly estimated from the intercept as lying between the values 800 and 1000 mM/kg/hr.

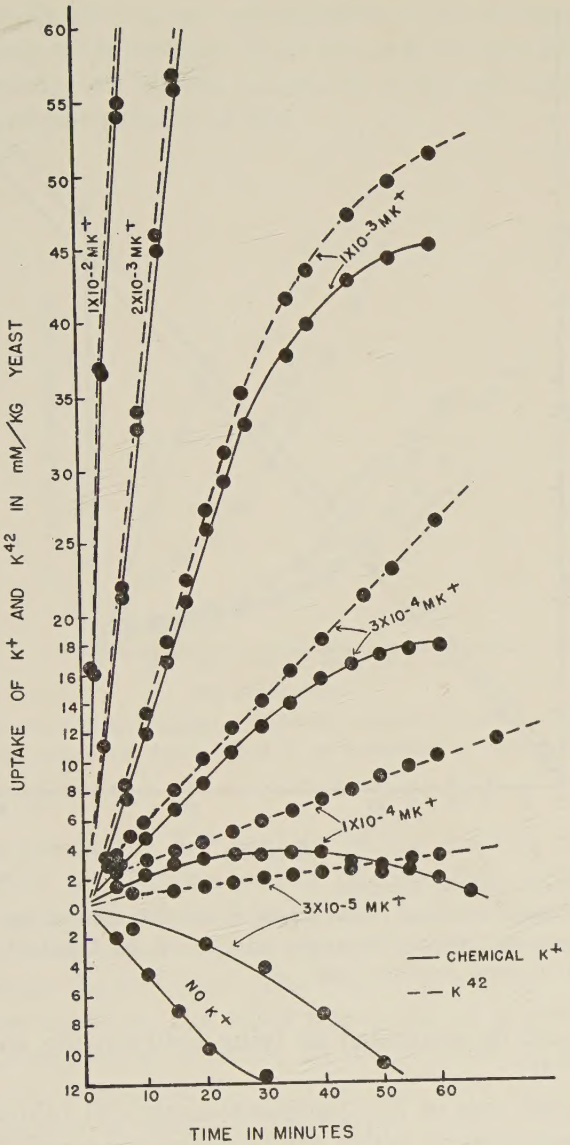


Fig. 4 Uptake or loss of $K^{42}$ and $K^{39}$ by fermenting yeast at different concentrations of potassium using the cell-column technique.

The amounts of yeast on the column were 600 mg for $K^+$ concentration below $1 \times 10^{-3}$ and 1200 mg for the higher $K^+$ concentrations. The glucose concentration was 0.05 M and pH 4.0.

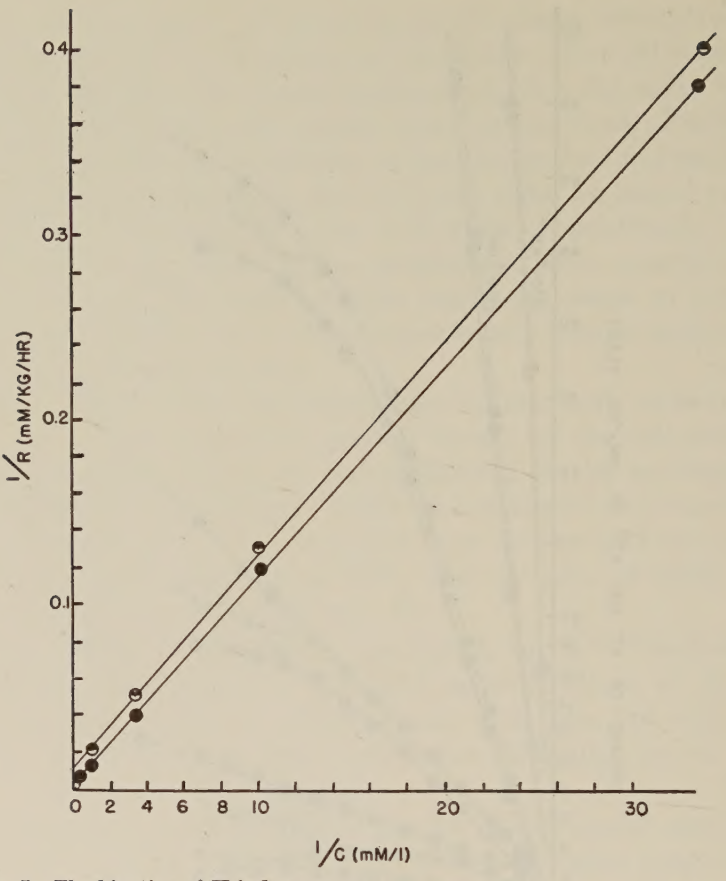


Fig. 5 The kinetics of K-influx.

Rates are taken from the initial slopes of the $K^{42}$ data of fig. 4. The solid circles represent all 6 slopes. The partly open circles, the 4 highest concentrations plotted on a 10 times enlarged scale.

The Km can be estimated as lying between the values 5 and $10 \times 10^{-3}$ M.

The movements of $K^{39}$ (chemical analyses) followed a more complicated pattern, which can be described briefly as follows: (a) with K-free media, there was a continuous loss of $K^{39}$ by the cells. (b) with low concentrations of $K^{39}$ ($3 \times 10^{-5}$ and $1 \times 10^{-4}$M) there was a reduced rate of loss, or a small uptake followed by a small loss. (c) with higher concentra-

tions of $K^{39}$ there was an uptake period which ceased after approximately 30 minutes.[5] Both the rate of uptake and the maximal amount of uptake increased progressively with in-increasing concentrations of $K^{39}$.

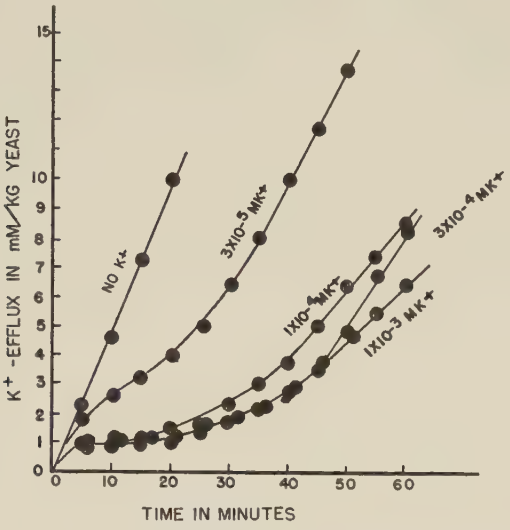


Fig. 6 The efflux of potassium at different concentrations of potassium. The data are calculated from figure 4 by substracting values for $K^{39}$ from values for $K^{42}$.

The changes in the $K^{39}$ content of the medium represents the net effect of K-influx minus the K-efflux. The rate of influx is directly determined by the $K^{42}$ measurements. Therefore the rate of efflux can be calculated by subtracting the $K^{39}$ data from the $K^{42}$ data. The results are given in figure 6. With K-free medium, the efflux proceeded at a constant rate of about 15 mM/kg of cells/hr. In the presence of potassium, the efflux was negligible for a time, but then increased to a rate comparable to that for the K-free media. The delay in the appearance of high rates of efflux was longer with suc-

[5] The flattening out of the curves for the two highest concentrations ($2 \times 10^{-3}$M and $1 \times 10^{-2}$ M) are not shown on figure 4 for lack of space. The maximal uptakes were 100 mM/kg and 140 mM/kg respectively. The latter value represents an 80% increase in K content of the cell.

ceedingly higher concentrations of potassium. With the highest potassium concentrations, the rate of efflux could not be determined. The rate of influx was so large compared to the rate of efflux that the difference between the $K^{42}$ and chemical data were within the limits of experimental error.

The net movements of potassium are determined by the relative rates of efflux and influx. At very low concentrations of potassium, the rate of efflux exceeds the rate of influx, and potassium is lost from the cell. At somewhat higher concentrations, the delayed appearance of K-efflux allows a net uptake followed by either a loss or no change. At the higher concentrations of potassium, the rate of K-influx is considerably greater than the rate of effiux and is the predominant factor.

## DISCUSSION

In the experiment of figure 4, a net uptake of potassium was observed when the extracellular concentration was as low as $1 \times 10^{-4}$ M. In view of the fact that the internal potassium concentration was 0.15 M per liter of cells, the potassium was absorbed against a concentration gradient of more than 1000 to 1. The uptake of potassium involves a stoichiometric exchange for $H^+$ (Rothstein and Enns, '46). The average pH of the cellular contents of fermenting yeast is 6.2 (Conway and Downey, '50), that in the medium was 4.5. Thus $H^+$ is moving against a gradient of about 50 to 1. The energy necessary for the active transport of the ions can be provided by either fermentation or respiration of glucose, or respiration of a variety of other substances (Orskov, '50). The nature of the transporting system is not specifically known, although mechanisms have been proposed (Conway, '54; Foulkes, '56). The asympototic relationship between the rate of potassium influx and the potassium concentration undoubtedly reflects the combination of potassium with a component of the transferring system, which becomes saturated at high K-concentrations. It is of some interest that the Km for the reaction of 0.5 to $1.0 \times 10^{-2}$ M is about the same as the dissociation

constant for the binding of potassium by fixed anionic groups of the cell surface of yeast (Rothstein and Hayes, '56), and also for the half maximal stimulation of fermentation by potassium (Rothstein and Demis, '53). The common constant for the three reactions, however, indicates only that potassium may be interacting with a similar chemical entity in each case, for evidence has previously been presented indicating that the reactions are not directly interrelated. Thus K-uptake can proceed even though potassium is blocked from the surface binding sites by bivalent cations such as $Mg^{++}$ or $UO_2^{++}$ (Rothstein, '56). Nor is stimulation of fermentation by potassium a necessary corollary to $K^+$-uptake (Rothstein and Demis, '53). From a chemical point of view, the surface binding sites have been identified as polyphosphates, or as polyphosphate-like compounds (such as nucleic acids) (Rothstein and Hayes, '56), which form similar complexes with potassium in vitro (Shack et al., '52; Van Wazer and Campanella, '50). The fermentation reactions stimulated by potassium are phosphorylation reactions which also involve a polyphosphate (ATP). On this basis it is suggested that the transporting reaction may involve an interaction of $K^+$ with a polyphosphate type of compound.

The K-influx is an active transport against the concentration gradient, whereas the efflux proceeds with the concentration gradient. Nevertheless, the variable factor which controls the net movements of potassium, particularly at lower concentrations of potassium, is the rate of efflux. The system behaves somewhat like a constant pump with a variable back-leakage. The lability of the efflux under various other conditions is discussed elsewhere (Rothstein and Bruce, '57).

## SUMMARY

The potassium metabolism of fermenting yeast cells was studied by a cell-column technique in which the medium was continuously changed. A steady-state condition could thereby be maintained at any desired potassium concentration.

The net movements of potassium are determined by the rate of K-influx and of K-efflux. Both rates are influenced by the potassium concentration. The rate of influx increases with K-concentration in an asymptotic relationship which can be fitted by the Michaelis-Menten equation with a Vm of about 1000 mM/kg of cells/hr., and a Km of $1 \times 10^{-2}$ M. At any particular concentration of potassium, the rate is constant for 30 to 60 minutes, then tends to flatten out. In contrast, the maximal rate of efflux is the same at all potassium concentrations, but the appearance of the efflux is delayed for a progressively longer time in the presence of progressively higher concentrations of potassium. In consequence the net absorption of potassium tends to flatten out or even reverse, especially at lower potassium concentrations. At high concentrations of potassium, the rate of efflux is not a controlling factor.

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# EFFECTS OF SULFHYDRYL BLOCKADE ON AXONAL FUNCTION [1]

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## INTRODUCTION

The following report describes experiments designed to test whether or not combination of sulfhydryl (SH) groups in nerve with non-physiologic compounds can cause axones to become inexcitable and to depolarize. It is of interest to examine this matter because of the reported importance of the SH group both for molecular level organization, and for metabolism in many biologically important systems.

Assuming that axons have certain properties in common with other cells, there are many findings reported in the literature which, by analogy, lead one to suspect that chemical masking of nerve SH groups would cause changes in macromolecular structure leading to functional aberrations which could be monitored and interpreted. For example, disulfide bridges are thought to be important in maintaining the structural stability of keratin (Astbury, '32). Reduction of the SH moieties of wool renders the fiber more readily stretched, and it is postulated that transformation of free SH groups to disulfide linkages accounts for the variety of stretch-states that can be established in wool (Burley, '55). The linkage between actin and myosin is thought to be by way of the SH groups, and, in addition, it has been claimed that the adensosine-triphospha-

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tase activity of myosin is directly associated with the SH group. Apparently adenosine triphosphate competes with actin for the SH sites of myosin, resulting in dissociation of the actomyosin molecule (Bailey and Perry, '47). At the whole cell level, it has been found that lysis of red blood cells is markedly increased by organic mercurial compounds which presumably react with the SH moieties of the cell (Benesch and Benesch, '54a). This perhaps suggests a role for SH groups with respect to the structural integrity of the cell surface. The sol-gel transformations of cleavage are inhibited by sulfhydryl-combining reagents, and therefore are thought to depend on the SH groups of the cytoplasmic proteins (Chalkley, '51). Hardening of the fertilization membrane seems also to involve disulfide linkages between protein (Runnström, '43).

The above then are some examples of the importance of SH groups at the level of macromolecular structure in biological systems. In addition, of course, compounds containing reactive SH groups play important roles in metabolism (Barron, '51). Emphasis in this report, however, is tentatively being placed more heavily upon the possible importance of SH group integrity for the structuro-functional rather than for the metabolic-functional relationship. This seems reasonable because the functional parameters studied, excitability, impulse propagation and resting potential appear, in some respects, not to be immediately associated with metabolism, but rather to be of an electro-kinetic nature, utilizing energy stored by metabolism over rather long time intervals, but immediately more dependent upon physicochemical structure of the cell (Brink, '52; Tobias, '52a, b; Hodgkin and Keynes, '53).

Earlier work on the effects of heavy metals and other inorganic compounds on nerve pointed indirectly to a relationship between SH groups and function. For instance, the arsenite ion produces conduction block, and the cupric ion has been shown to depolarize in sheath intact frog nerve, after a long period of exposure (Schmitt, '35; Lorente de No, '47).

In desheathed frog nerve preparations, solutions of cadmium, copper, mercury, silver and uranyl ions in concentrations of the order of 1.0 mM produce conduction block of rapid onset which is reversible by washing in 1.0 mM solutions of cysteine or glutathione (del Castillo-Nicolau, '51).

Though suggestive, these experiments did not prove that the toxic agents used had their effects on the nerve by way of combining with cellular SH groups. First of all, the heavy metals are not selective for SH groups, and could have produced their effects by reacting with hydroxyl, carboxyl, guanido, or sulfhydryl groups (Olcott, '47). Secondly, prevention or reversal of the heavy metal ion effects by adding SH compounds, such as cysteine or glutathione, might have occurred simply because these compounds were able to compete successfully with carboxyl, hydroxyl or guanido groups for the heavy metal.

The problem then was to design experiments which would allow a somewhat more certain assessment of the relationship of function to the chemical status of the nerve SH groups. The following general approach was adopted: whole nerves and axons were to be treated with reagents whose degree of specificity for SH group combination could be anticipated in terms of an informed guess. Thus it was decided, on the basis of information in the literature, that n-ethylmaleimide (NEM) and p-chloromercuribenzoic acid (PCMB) would preferentially react with SH groups (Friedman, '49; Barron, '51; Snoke and Bloch, '52; Barrnett and Seligman, '54), whereas copper, used as the chloride, might very well react more non-specifically with other negatively charged sites, and it was assumed that functional changes would be produced by these substances. Now then, if these functional changes could be prevented and/or reversed by innocuous competing substances containing reactive SH groups, as glutathione or cysteine, but not by ones containing hydroxyl, carboxyl, amino, or guanido groups alone, then the notion that the original agent was acting by combining with SH groups in the cell would be strengthened.

On the other hand, if the original agent were equally well counteracted by the hydroxyl, carboxyl, amino, or guanido-containing competitors, then it certainly could not be concluded that the effect in the first place was due to the blockade of nerve SH groups.

## METHODS

Two sources of nerve tissue were employed; frog sciatic nerve and the isolated giant axon of the lobster (*Homarus americanus*) ventral nerve cord, prepared after the technique of Tobias and Bryant ('55). Attempts were made to use *Panilirus interruptus,* but the short neural connections to the central ventral ganglion yielded axon lengths too short to use.

Standard techniques were used to monitor electrical activity. Resting potentials were measured by axonal impalement with a glass micropipette filled with 3M KCl. Ling's Ringer solution VII for frog nerve ('49), and filtered sea water for the lobster axon were the physiologic solutions used. The laboratory temperature did not fluctuate more than two degrees during any one experimental day. The pH of the ambient fluid in the nerve chamber was determined at the end of each experiment with a glass electrode. A wide range of reagent concentrations was tested. The concentrations finally used were selected because the time lapses involved were convenient.

In terms of the overall experimental plan outlined in the introduction, many small organic molecules which possess reactive groups similar to those found in protein were considered for use as competitors. Among these were glycine, glycine anhydride, glycylglycine, serine, arginine, salicylic acid, cysteine and glutathione. In concentrations ten to one hundred times the concentration of the sulfhydryl-combining reagents, these substances had little or no effect on the frog nerve action potential despite two or more hours of exposure. Cysteine, in 2.0 mM concentrations, caused a slow but steady increase in the threshold for excitation. In lobster axons serine occasionally caused block after a sixty minute exposure.

Experiments with the isolated lobster axon were always begun with a half-hour control period to determine the effects of the dissection. If no deteriorative changes appeared in this time, the test materials were added, and the conduction velocity and threshold were followed until block occurred; then the nerve was impaled and the resting potential followed to extinction. The potentially protective substance was always added at least one hour before the SH-combining reagent. Impalement before the addition of the sulfhydryl-combining agent or before conduction block occurred seemed to accelerate the onset of block as well as the rate of reduction of the resting potential. This effect was localized to the impalement site since reimpalement immediately adjacent to the deteriorated site indicated no such change. The time for the reduction of the resting potential therefore was not determined with a single impalement but with a series of impalements. A potential of twenty millivolts was taken arbitrarily as a practical endpoint for resting potential reduction.

## RESULTS

Results are shown in tables 1 and 2.

The three reagents, $CuCl_2$, NEM and PCMB are seen all to have produced inexcitability in whole frog nerve and in lobster axons. Using the lobster axons, the additional observation was made that the onset of inexcitability was followed by depolarization.

Early experiments showed that frog nerve block by $CuCl_2$ could be prevented by all the competitors used except glycine anhydride which has no reactive groups. Apparently the cupric ion reacted rather non-selectively with carboxyl, hydroxyl, amino, guanido and SH groups as was anticipated. For this reason it was decided that further experiments with the Cu ion would not yield much information apropos specifically the nerve SH groups. Therefore subsequent experiments were done only with NEM and PCMB.

It was then found that among all the substances used only glutathione or cysteine protected frog nerve against NEM or

PCMB. Experiments with lobster axons confirmed the protection by these substances. In the case of frog nerve there was some prolongation of the time to block when glycylglycine, arginine, or serine were used as competitors, but no such partial protection was seen with lobster axons. The reason for this difference is not immediately apparent.

These data then strongly suggest that blockade of nerve SH groups results in inexcitability and secondary depolarization.

TABLE 1

*Effects of reagents used on frog nerve*

| SULFHYDRYL REAGENT | | COMPETITOR FOR SH REAGENT | | EFFECT ON FROG NERVE | |
|---|---|---|---|---|---|
| Substance | Conc. mM | Substance | Conc. mM | No. of expts. | Time-to-block in minutes [1] |
| NEM | 2.0 | | | 9 | 21 ± 9 |
| PCMB | 0.002–02 | | | 8 | 4 ± 1 |
| $CuCl_2$ | 0.2 | | | 6 | 16 ± 7 |
| $CuCl_2$ | 0.4 | | | 4 | 6 ± 2 |
| NEM | 1.7 | glycine anhydride | 20–100 | 6 | 25 ± 9 |
| PCMB | 0.17 | glycine anhydride | 20 | 6 | 3 ± 1 |
| $CuCl_2$ | 0.17 | glycine anhydride | 20 | 3 | 16 ± 3 |
| NEM | 1.7 | glycine | 20 | 6 | 19 ± 7 |
| PCMB | 0.17 | glycine | 20 | 5 | 5 ± 2 |
| $CuCl_2$ | 0.17 | glycine | 20 | 5 | NB(118–134) [2] |
| NEM | 2.0 | glycylglycine | 20 | 5 | 39 ± 4 |
| PCMB | 0.2–0.4 | glycylglycine | 20 | 9 | 30 ± 4 |
| $CuCl_2$ | 0.2–0.5 | glycylglycine | 20 | 3 | NB(70–125) |
| NEM | 1.7–2.0 | arginine | 20 | 7 | 23 ± 5 |
| PCMB | 0.17–0.2 | arginine | 20 | 8 | 17 ± 5 |
| $CuCl_2$ | 0.17 | arginine | 20 | 6 | NB(60–130) |
| NEM | 1.4–2.0 | serine | 20 | 5 | 22 ± 12 |
| PCMB | 1.7–2.0 | serine | 20 | 5 | 10 ± 9.8 |
| $CuCl_2$ | 1.7–2.0 | serine | 20 | 6 | NB(72–159) |
| NEM | 2.0 | salicylic acid | 20 | 8 | 28 ± 16 |
| PCMB | 1.7 | salicylic acid | 20 | 5 | 9 ± 12 |
| $CuCl_2$ | 1.7–2.0 | salicylic acid | 20 | 6 | NB(61–146) |
| NEM | 1.7 | glutathione | 20 | 4 | NB(180–303) |
| PCMB | 0.17 | glutathione | 20 | 9 | NB(150–210) |
| NEM | 2.0 | cysteine | 20 | 6 | NB(180) |
| PCMB | 0.2 | cysteine | 20 | 6 | NB(180) |

[1] Average ± S.E.

[2] NB means no block occurred in this time lapse.

Attempts were also made to reverse the effects of the sulfhydryl-combining reagent once established. Glutathione was found to reverse $CuCl_2$ block in frog nerve (three experiments) (see also, del Castillo-Nicolau, '51), but alone it failed to reverse the action of NEM or PCMB (ten experiments). Since PCMB was known to form an electrostatic bond, it was felt that its effects might be reversed if it could first be dissociated from the nerve by high pH. By way of control, it was

TABLE 2

*Effects of reagents used on lobster axons*

| RE-AGENT | CONC. mM | COMPETITOR FOR SH REAGENT | | NO. OF EXPTS. | TIME TO BLOCK [1] | TIME TO DEPOLARIZE TO 20 mV |
|---|---|---|---|---|---|---|
| | | Substance | Conc. mM | | | |
| NEM | 1.0 | | | 9 | 10 ± 5 | 90 ± 16 |
| | 2.0 | | | 6 | 7 ± 3 | 27 ± 18 |
| PCMB | 0.045–0.07 | | | 7 | 3 ± 1 | 23 ± 6 |
| | 0.2 | | | 5 | 1 ± 1 | 6 ± 2 |
| $CuCl_2$ | 0.1 | | | 9 | 11 ± 7 | 55 ± 27 [2] |
| NEM | 2.0 | glycylglycine | 20 | 7 | 11 ± 5 | |
| PCMB | 0.2 | glycylglycine | 20 | 5 | 2 ± 1 | |
| PCMB | 0.2 | arginine | 20 | 5 | 2 ± 2 | |
| PCMB | 0.2 | serine | 20 | 5 | 2 ± 1 | |
| NEM | 2.0 | glutathione | 20 | 6 | NB(60) [3] | |
| PCMB | 0.2 | glutathione | 20 | 6 | NB(60) | |
| NEM | 2.0 | cysteine | 20 | 6 | NB(60) | |
| PCMB | 0.2 | cysteine | 20 | 6 | NB(60) | |

[1] Time in minutes ± standard error.

[2] Based on three of the nine experiments.

[3] NB, no block in the time in minutes stated.

first shown that frog nerve could survive exposure to a phosphate buffered solution at pH 10–11 for approximately two minutes, followed by washing in ordinary Ringer solution, without irreversible damage. Such treatment alone did not produce reversal of PCMB block (six experiments), but a combination of glutathione (2.0 mM) and a pH of ten to eleven for one or two minutes, followed by washes in ordinary Ringer solution resulted, in reversal in some cases (4 out of 9). Reproducible results were not achieved until

freshly prepared glutathione solutions were used. Then, in twelve experiments recovery, roughly classified as good in terms of the A elevation of the compound action potential, was found in eight cases and, as poor, in four. Total recovery was never achieved. In the case of NEM, similar treatment of frog nerve with solutions of high pH alone (three experiments), or with glutathione in the presence of high pH (six experiments) did not produce reversal of block. This might have been expected, since the thiol bond with NEM is covalent in nature, and requires boiling with Raney nickel to effect chemical separation (Marrian, '49). Attempts were made to reverse the effects of NEM and PCMB in the lobster axons, but without success. The lobster axon did not tolerate a pH above 9.5 (glycine-NaOH buffer), and glutathione alone (six experiments) was ineffective.

## DISCUSSION

Several matters in the foregoing warrant brief discussion.

The data show that NEM is not as active as PCMB in producing inexcitability in nerve; i.e., concentrations of NEM 10 to 40 times as great as those of PCMB still require 2 to 5 times as long as the PCMB to produce the functional changes observed. This discrepancy is apparently not to be explained in any obvious way simply in terms of penetration because of the following observation: in each interganglionic stretch of the nerve cord there are two connectives, each surrounded by a connective tissue sheath. It is usual to isolate the giant axon in one, leaving the other intact for mechanical support. The second giant axon can also be isolated, however, and it was found that this second axon was blocked by NEM when the connective tissue sheath was still intact, but not by PCMB (5 experiments). That is NEM was apparently able to penetrate the connective tissue sheath, but PCMB was not. On these grounds it might be suspected that NEM would be more effective rather than less so as was found. Nor is it possible to explain the difference in terms of lipid solubility since too few pertinent data are available.

Another point to consider concerning this apparent activity difference has to do with the fact that there are three types of differently reactive SH groups in proteins, the reduced or freely reactive, the sluggishly reactive, and the masked (Benesch and Benesch, '54b; Calvin, '54). NEM reacts with a single freely acting group by adding the hydrogen and sulfur moieties across its double bond to form an irreversible covalent bond. PCMB forms a reversible electrostatic bond with a single thiol of both the freely and the sluggishly acting types. Since one can say nothing about the matter of accessibility, it may be that the greater reactivity of PCMB with sluggishly reactive SH groups determines its greater effectiveness. Actually the problem is not yet clarified.

In the introduction, it was suggested that a major role of the SH group is played with respect to the structure of the cell. A possibility for explaining the functional changes resulting from SH group blockade is that the structure of the surface is altered as a result of removal of SH groups available for cross bonding. Apparently there are at least two types of protein gels, one in which hydrogen bonding is dominant, and one in which disulfide bonding is dominant, and it has been postulated that formation of the disulfide bonded gel might be catalyzed by free SH groups (Huggins, '50, '51). Conceivably, action potential generation and recovery might require that the cell surface be able to form and break such a gel. It is known, for instance, that high hydrostatic pressure which interferes with sol-gel transformations can reversibly block nerve (Grundfest, '36). The change would involve a shift from a surface structure in which the disulfide bond is statistically dominant during either the resting or active state to a structure in which the hydrogen bond was statistically dominant during the opposite phase. The hydrogen bond type of gel, where studied, has been described as firm and opaque (Huggins, '50). During the impalement experiments, the impression was gained that the axon became more opaque and resistant to impalement after sulfhydryl blockade had occurred.

## CONCLUSIONS

1. The data strongly suggest that blockade of the sulfhydryl group in lobster and frog nerve results in the loss of excitability and in reduction of the resting potential.

2. The sulfhydryl-combining reagents, n-ethylmaleimide and p-chloromercuribenzoic acid react in a relatively selective fashion with the sulfhydryl group of nerve.

3. The blocking action of p-chloromercuribenzoic acid can be reversed. To date it has not been possible to reverse the blocking action of n-ethylmaleimide.

4. A possible role of the sulfhydryl group in the relationship between structure and function in nerve is discussed.

## ACKNOWLEDGMENTS

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# INTERACTION OF INSECT ACTOMYOSIN WITH ADENOSINE TRIPHOSPHATE [1]

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SEVEN FIGURES

## INTRODUCTION

Since Engelhardt and Ljubimova ('39) discovered the adenosine triphosphatase (ATPase) activity of myosin, the structural protein of muscle, and a little later A. Szent-Györgyi and his collaborators elucidated the remarkable interaction of actomyosin with adenosine triphosphate (ATP) (cf. Szent-Györgyi, '45), a great number of papers in muscle biochemistry have centered on these phenomena, and the current physicochemical theories of muscular contraction have been founded on the basis of these investigations, but none is yet established (see reviews: Szent-Györgyi, '51; Morales et al., '55; Weber and Portzehl, '52, '54; and Tonomura, '56). These numerous studies were chiefly made with rabbit skeletal muscle.

In invertebrates, insects have the most specialized striated muscle, but researches on the contractile protein from insect muscle have only recently commenced and the information available is rather incomplete. Gilmour and Calaby ('53) first reported the viscosity change with ATP, and ATPase activity in locust muscle, and Maruyama ('54a, b) studied the enzymatic properties of actomyosin ATPase in more detail in several species of insects. This work has shown that insect

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actomyosin is essentially similar to that of rabbit with respect to the response to ATP. Correspondingly, ATP from insect muscle has been clearly identified with that from rabbit tissues (Albaum and Kletzkin, '48; Calaby, '51).

The present article is concerned with some physiocochemical observations on the interaction between the actomyosin from honeybee muscle and ATP, including superprecipitation, viscosity change, light-scattering change, and kinetics of ATPase action, following the procedures of Tonomura's school (see Tonomura '56).

## EXPERIMENTAL

*Material.* The adult workers of the honeybee, *Apis mellifera L.* of the age of 10-20 days were collected from a bee-hive and used as source material.

*Preparation of actomyosin.* The active bees were immobilized by cooling them for 30 min. at 0°C and then the thorax was cut out, removing the head, abdomen and legs. In each case about 6 to 7 hundred thoraces (about 20 gm wet weight) were ground little by little in 200 ml of the cold Weber-Edsall solution (0.6 M KCl–0.04 M $NaHCO_3$–0.01 M $Na_2CO_3$) with a glass-rod homogenizer of the Potter type. The suspension was kept for 3 hours at 0°C with occasional agitation, and then centrifuged after the filtration through several layers of gauze. Actomyosin was purified as previously described (Maruyama, '54a), by repeating 3 times the dilution procedure. Actomyosin was finally dissolved in 0.6 M KCl, insoluble matter was removed by centrifugation, and the supernatant solution was filtered through several layers of gauze.

*ATP.* Disodium salt of ATP ("99% pure") was purchased from the Nutritial Biochemicals Co., Cleveland, Ohio. The ATP was dissolved in distilled water, neutralized to pH 7 with 0.6 N KOH and stored at approximately —10°C, until used. Enzymatic analysis of this ATP sample, using insect actomyosin ATPase (cf. fig. 5) showed a very high purity. Inorganic orthophosphate (P) was determined by the Behrenblum-Chain method, as modified by Takahashi ('54).

*Ultraviolet absorption spectra.* A Shimazu spectrophotometer was employed, using a cell of 1 cm of light path.

*Superprecipitation.* In a short test tube immersed in a water bath at 20°C, superprecipitation on addition of ATP was observed by eye.

*Viscosity change.* A viscosimeter of the Ostwald type was used. Usually 3.5 ml of actomyosin solution in 0.6 M KCl (pH 6.4) and 0.1 ml of 0.12 M $MgCl_2$ or $CaCl_2$ or water were pipetted into the viscosimeter and placed in a water bath at 16°C, until the flow time became constant. Then 0.2 ml of ATP were very rapidly introduced and mixed.

*Light-scattering change.* Intensity of light scattered by actomyosin dissolved in 0.6 M KCl was measured at 90° by an electron multiplier-μ ammeter, using an apparatus described by Tonomura et al. ('53). For routine assay, 0.2 ml of ATP were rapidly introduced into a mixture of 14.5 ml of actomyosin solution and of 0.3 ml of 0.125 M $MgCl_2$, at pH 6.4 at 22°C.

*ATPase tests.* ATPase activity was measured in a standard system consisting of 1.0 ml of 0.15 M histidine buffer, containing 0.3 M KCl, 1.0 ml of ATP, 0.3 ml of 0.1 M $CaCl_2$, 0.6 ml of actomyosin solution and 0.1 ml of water. Usually the mixture was incubated for 30, 60 and 90 seconds at pH 6.8 at 12°C, with constant agitation. Some measurements were done at pH 6.0 at 32°C. In each case the zero-order rate constant was calculated. In one experiment, time activity course was followed up to 50 min. at pH 6.0 at 37°C. The enzyme action was stopped by adding 1.0 ml of 10% trichloroacetic acid. Controls without enzyme or substrate were always run in parallel.

*Determination of P and N.* 1.0 ml of aliquot of the deproteinized filtrate was assayed for P by the method of Youngburg and Youngburg ('30), using an electrophotometer. The duplicate assays were run to check the experimental error below 5%. Only in the one case (fig. 5), more accurate method of P estimation was employed in order to confirm that only the terminal P bond of ATP was hydrolyzed by actomyosin-ATPase.

Nitrogen was determined by a Kjeldahl procedure and a factor of 6 was used to calculate the protein content.

Glass-distilled water was used throughout.

## RESULTS

*Notes on honyebee actomyosin.* The actomyosin solution prepared from honeybee thoracic muscle and dissolved in 0.6 M KCl was very turbid and yellowish brown in color, similar to that from locust thoracic muscle reported by Gilmour and Calaby ('53). It was quite soluble in 0.6 M KCl and easily precipitable below 0.1 M KCl.

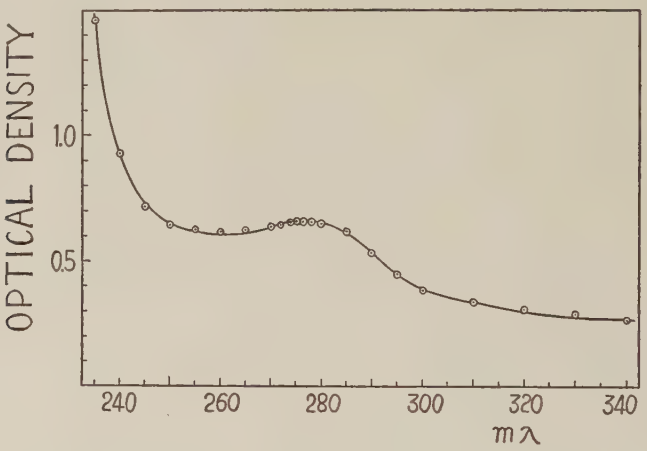


Fig. 1 Ultraviolet absorption spectra of insect actomyosin solution in 0.6 M KCl. 0.045 mg nitrogen per ml.

Ultraviolet absorption spectra, shown in fig. 1, indicated a slight peak and trough of absorption around 275 and 260 mμ of wave length, which are essentially the same as in rabbit actomyosin (Tarver and Morales, '51). It is to be noted that the extinction coefficient at 275 mμ (0.1% nitrogen; 1 cm light path) is much higher (about 13) than that of rabbit (which is about 3–4). The ratio of the extinction coefficient at 270 mμ to that at 260 mμ is about 1.05.

*Superprecipitation.* ATP caused a violent flocculent precipitation of insect actomyosin, as clearly seen in fig. 2. At

20°C at pH 6.9 (veronal-acetate buffer), in the presence of 0.1 M KCl such a precipitation occurred within 1 min., after the addition of $1 \times 10^{-4}$ M ATP. After a few minutes, it contracted into a small plug (see fig. 2, B). This is quite similar to the phenomenon referred to "superprecipitation" in rabbit

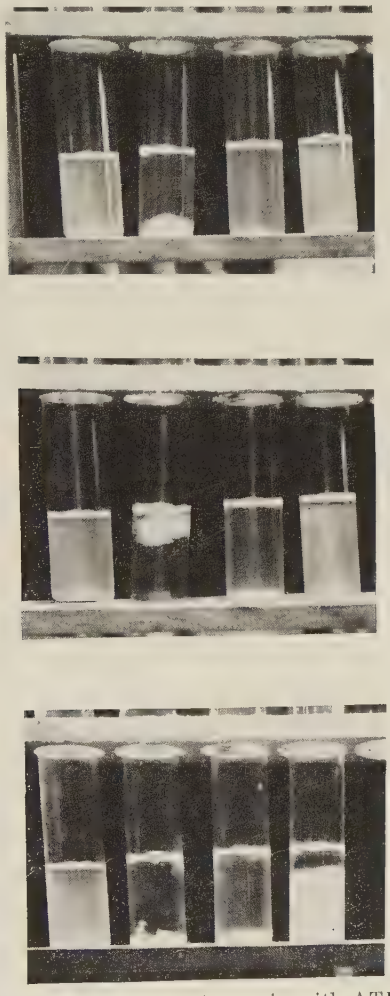

Fig. 2 Superprecipitation of insect actomyosin with ATP. 0.1 M KCl; 0.5 mg protein per ml. Left to right: control; plus $1 \times 10^{-4}$ M ATP; plus $1 \times 10^{-4}$ M $MgCl_2$ and ATP; and plus $CaCl_2$ and ATP. 2 (A), 30 (B) and 60 (C) min. after the addition of ATP. Kindly photographed by Dr. H. Matsumiya.

actomyosin (cf. Szent-Györgyi, '45). It was most remarkably observed in the presence of 0.09–0.11 M KCl and in more than 0.15 M and less than 0.05 M KCl, it was not so clearly recognized. Even in the presence of 0.1 M KCl, addition of $1 \times 10^{-4}$ M $CaCl_2$ or $MgCl_2$ seemed to retard the apparent superprecipitation, as shown in fig. 2. Particularly Ca ions were likely to be more inhibitory than Mg, in good agreement with observations on rabbit actomyosin (Szent-Györgyi, '45; Spicer and Bowen, '51).

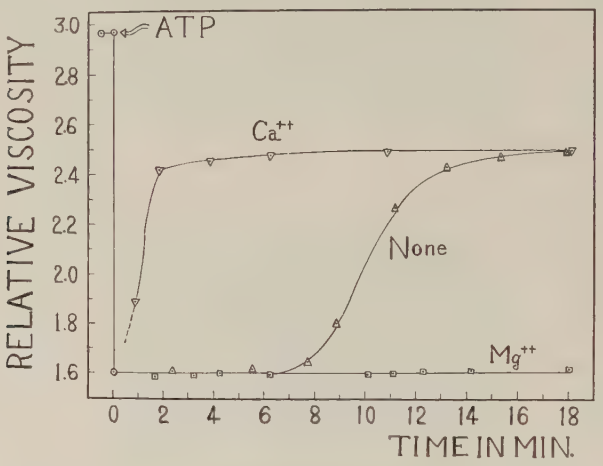


Fig. 3 Viscosity drop of insect actomyosin solution with ATP. 0.6 M KCl; 2.7 mg protein per ml; $1.7 \times 10^{-4}$ M ATP; $3 \times 10^{-3}$ M $CaCl_2$ or $MgCl_2$, when added.

*Viscosity change.* Insect actomyosin solution in 0.6 M KCl possessed a high viscosity, $Z$, the viscosity number ($Z = \ln \tau\,\mathrm{rel}/c$; $c$ = protein concentration, g $L^{-1}$) being 0.40. Addition of ATP immediately caused a marked viscosity drop: the viscosity number in the presence of $1.7 \times 10^{-4}$ M ATP ($Z_{ATP}$) was 0.17. The ATP sensitivity, defined as $\frac{Z - Z_{ATP}}{Z_{ATP}} \times 100$ by H. H. Weber's school (cf. Weber and Portzehl, '52) was about 135%, and thus comparable to that of rabbit skeletal actomyosin.

The reduced viscosity with ATP slowly rose again to a considerable extent. Figure 2 shows a typical viscosity change

with ATP, where Mg ions retarded the recovery process and Ca promoted it. The final recovery in any case was around 60% of the original value and the complete recovery could not be observed even after a prolonged time, in contrast to the result obtained with locust actomysin (Gilmour and Calaby, '53). The present result is very similar to that obtained with rabbit actomyosin (Mommaerts, '48). The effects of Ca and Mg ions on the recovery process may be related to those on the ATPase action of actomyosin ATPase: Ca greatly activates the ATP breakdown by actomyosin ATPase and Mg inhibits it. However the recovery process may not solely be due to the breakdown of the added ATP (see Tonomura, Yagi and Matsumiya, '55).

Addition of $3.0 \times 10^{-4}$ M inorganic pyrophosphate, in the presence of $3 \times 10^{-3}$ M $MgCl_2$, also resulted in viscosity drop: $Z$ lowered from 0.39 to 0.19. No recovery process was observed. In the absence of Mg, no viscosity change was observed, nor did a viscosity change occur on adding Ca, in good agreement with the results obtained with rabbit actomyosin (Mommaerts, '48).

*Light-scattering change.* On addition of ATP, the intensity of scattered light slightly dropped, owing to some kind of deformation of actomyosin particles with ATP (cf. Morales et al., '55; Tonomura, '56). The maximum drop of scattered light intensity was only 7%, much smaller than 30 to 40% of rabbit (Tonomura, Watanabe and Yagi, '53) or of pecten (Tonomura, Yagi and Matsumiya, '55). This small percentage is rather of the same order of magnitude as that for swine oesophagus actomyosin (5%; Tonomura and Sasaki, '57). Although tested in three separate preparations of different protein concentrations, the same small value was observed in each case.

As in rabbit actomyosin (Tonomura et al., '53), in the presence of $2.5 \times 10^{-3}$ M $MgCl_2$, the drop of intensity of scattered light was proportional to the concentration of the ATP added until its value reached the constant maximum value. The latter value was linear with the concentration of the actomyosin

used as the sample. From figure 4, the weight of insect actomyosin which combined with one mole of ATP can be calculated, giving a value of $4.0 \times 10^5$ g. This value is a little larger than that of rabbit preparations ($1.9 \times 10^5$ g; Tonomura et al., '53).

*Kinetics of ATPase action.* Actomyosin from honeybee thoracic muscle possessed a powerful ATPase action in a strict sense, removing only the terminal P group from ATP in the presence and absence of Ca ions, as illustrated in fig. 5. In the presence of Mg, however, although the initial rate was

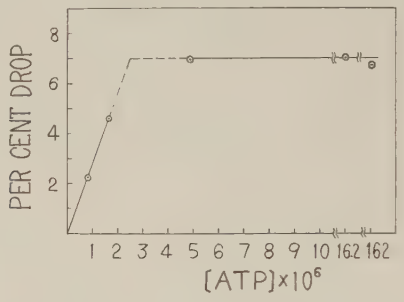


Fig. 4 Relationship between the grade of the light-scattering drop and the concentration of ATP added. 0.6 M KCl; 1 mg protein per ml; $2.5 \times 10^{-3}$ M $MgCl_2$.

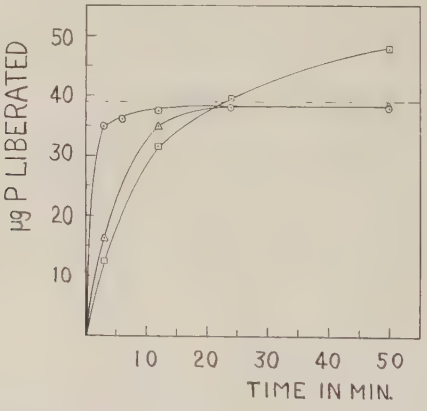


Fig. 5 Time activity course of insect actomyosin ATPase. 0.2 M KCl; 0.75 mg protein; pH 6.0; 37°C; ○ – – – ○: $6.6 \times 10^{-3}$ M $CaCl_2$; □ – – – □: $MgCl_2$; △ – – – △: none added. – – – –: the value of ½ of the labile P of the added ATP.

lower, more than the half of all the labile P was liberated in a prolonged incubation. This situation is the same as that observed in locust muscle (Gilmour and Calaby, '53). The effect of Mg on the prolonged enzyme action may be due to the Mg-activated apyrase action slightly contaminated in the actomyosin preparation, as reasonably explained by the same authors.

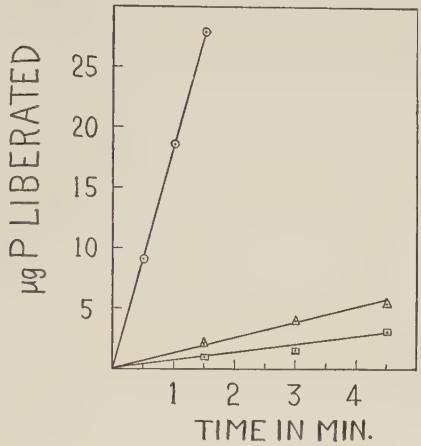


Fig. 6 Initial velocity of insect actomyosin ATPase. 0.2 M KCl; 0.25 mg protein; pH 6.0; 32°C; $1 \times 10^{-3}$ M ATP; ○---○; 0.01 M $CaCl_2$; □---□: $MgCl_2$; △---△: none added.

In a short time of incubation, Ca greatly stimulated ATPase activity and Mg inhibited it, as clearly demonstrated in fig. 6. Mg also markedly inhibited the activating effect of Ca. In the presence of the sufficient amount of ATP, the ATPase action proceeded in accordance with a zero order kinetics in all these cases.

The relation between the initial rate (v) of the ATPase action and the initial concentration of ATP (S) is expressed in the Michaelis-Menten formula:

$$v = \frac{Vmax}{1 + \frac{Km}{[S]}} \quad \text{or} \quad \frac{1}{v} = \frac{1}{Vmax}\left(1 + \frac{Km}{[S]}\right)$$

therefore the following scheme for the enzyme action may be given:

$$AM + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AM \cdot S \qquad AM \cdot S \xrightarrow{k_2} AM + P$$

hence,

$$Km = \frac{k_{-1} + k_2}{k_1}, \qquad Vmax = k_2 [AM]$$

Here, AM, [AM], AM · S and P represent actomyosin, its total concentration, ATP-actomyosin complex and the reaction product respectively and $k_s$ are the velocity constants for each reaction step.

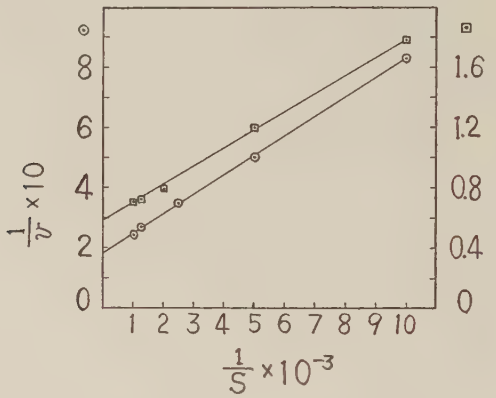


Fig. 7 Effect of the substrate concentration on the initial velocity of insect actomyosin ATPase. 0.2 M KCl; 0.01 M $CaCl_2$; ○---○: 0.5 mg protein; pH 6.8; 12°C (ordinate: left); □---□: 0.25 mg protein; pH 6.0; 32°C (ordinate: right). S: ATP concentration. v: μg P liberated for 1 min.

The Michaelis constant, Km and the maximum velocity, Vmax were $3.4 \times 10^{-4}$ M $L^{-1}$ and $5.9 \times 10^{-6}$ M $sec^{-1}$ $g^{-1}$ at pH 6.8 at 12°C in the presence of $1 \times 10^{-2}$ M $CaCl_2$ and 0.2 M KCl, as calculated from fig. 7. Knowing the unit weight of insect actomyosin as $4.0 \times 10^5$ g from the light-scattering work, we can also calculate the rate constant, $k_2$ as 2.4 $sec^{-1}$. These values are the same order of the magnitude as those obtained for actomyosin ATPase from various animals under the same

*in vitro* conditions (see table 1). Under the more suitable conditions for insect actomyosin ATPase, such as at pH 6.0 at 32°C (cf. Maruyama, '54a), the following kinetic constants were obtained: Km: $2.0 \times 10^{-4}$ M $L^{-1}$; Vmax: $38 \times 10^{-6}$ M $sec^{-1}$ $g^{-1}$; and $k_2$: 15.2 $sec^{-1}$; respectively.

## DISCUSSION

Gilmour and Calaby ('53) precisely showed that actin is more easily extractable in locust muscle than in the rabbit one, so that myosin B of Szent-Györgyi ('45) or natural actomyosin can be gained by extracting insect muscle suspension in strong salt solution for only 2 hours. This fact was confirmed by testing "shrinking of an actomyosin thread with ATP" (see Szent-Györgyi, '45; '51) in the 2 hour-extracted preparations from thoracic muscles of several insects (Maruyama, '54a). In the present study also, the 3 hour-extracted sample from honeybee muscle indicated the specific characteristics of actomyosin, not of myosin proper.

In the preceding pages, some physiocochemical aspects of the interaction of insect actomyosin with ATP were described

TABLE 1

*Comparison of actomyosin ATPase from various animals*

| ANIMAL | TYPE OF MUSCLE | VMAX ($\mu$M $sec^{-1}$ $g^{-1}$) | ($10^5$g) UNIT WT. | $k_2$ ($sec^{-1}$) | Km (mM) | AUTHOR |
|---|---|---|---|---|---|---|
| Honeybee | thoracic | 38 * | 4.0 | 15.2 * | 0.20 * | This paper |
| | | 5.9 | | 1.2 | 0.30 | |
| Rabbit | skeletal | 6 | 1.9 | 1.2 | 0.15 | Tonomura, Watanabe and Yage, '53 |
| Swine | oesophagus | 3.9 | 10 | 3.9 | 0.53 | Tonomura and Sasaki, '57 |
| Pecten | fast adducter | 9.6 | 3.9 | 3.7 | 0.41 | Tonomura, Yagi and Matsumiya, '55; '56 |
| | slow adducter | 3.2 | 12.2 | 3.9 | 0.45 | |

Conditions: 0.01 M $CaCl_2$; 0.2 M KCl; pH 6.8 – 7.0; 12°C, except the marked value (*); pH 6.0; 32°C.

in comparison with those in rabbit actomyosin, and these showed no fundamental difference between the contactile proteins from both muscles with respect to the interaction with ATP. Especially, superprecipitation, viscosity drop and ATPase action are both qualitatively and quantitatively similar to each other. Only a quantitative difference was found in the smaller percentage of light-scattering change with ATP in insect actomyosin.

The present preparations, though purified by the conventional method,[3] might contain some minor impurity of large size of particles, which gave an appreciable effect on the intensity of scattered light, as well as on that of ultraviolet absorption spectra. In the latter case, the scattered light may interfere with the absorption spectra by increasing the apparent optical density, and so be responsible for the unusually high extinction coefficient of the ultraviolet absorption spectra. However, such a contamination may be very small in amount, since a great viscosity drop with ATP was observed. The impurity might be to the fact the whole thorax was employed as the starting material for actomyosin preparation.

Much attention has been attached to the actomyosin ATPase activity, since it is assumed to play an important role in the energy-supplying mechanism for muscular contraction (cf. Weber and Portzehl, '54; Morales et al., '55; Tonomura, '56). In insect muscle, the ATPase property of actomyosin has been investigated in some detail. According to the data hitherto reported (Gilmour and Calaby, '53; Maruyama, '54a),[4] all the actomyosins from thoracic muscles of more than ten species of insects belonging to different orders of classification have

[3] As pointed out by a referee, if the preparation would be clarified by high-speed centrifugation, a remarkable drop in the intensity of scattered light would be expected to occur on the addition of ATP. The detailed work on the light-scattering of insect actomyosin will be done in near future along such a line.

[4] I am much indebted to Dr. D. Gilmour of Canberra for his helpful personal communications on this subject. His results, including the unpublished ones, are in good agreement with mine. Furthermore, according to his unpublished investigation, actin-free myosin ATPase showed a similar response to metal ions as actomyosin in locust muscle.

a similar ATPase property and the following characteristics in contrast to that in rabbit skeletal muscle:

(1) There are two pH optima, a sharp true one at pH 5.9–6.0 and another lower apparent one around pH 8.5–9.2 in the presence of appropriate concentrations of KCl and $CaCl_2$ at the physiological temperature.

(2) No appreciable activation by Mg ions is observed, depending upon the K ion concentration, other than due to the minor contamination of the Mg-activated apyrase.

(3) Insect ATPase is not so heat-labile as the rabbit one.

It is of great interest to compare the grade of the ATP-splitting ability of insect actomysin with that of the actomyosins of other animals, in view of the very rapid function of insect indirect flight muscle. As summarized in table 1, there is no great difference in the ATPase activity of different types of muscles of several animals at pH 6.8 at 12°C in the presence of appropriate activators ($1 \times 10^{-2}$ M $CaCl_2$; 0.2 M KCl). The Michaelis constant, Km is also of the similar magnitude each other. The honeybee cannot fly at temperatures as low as 12°C, so that, in addition, a temperature of 32°C at which the bee flies very quickly, was chosen for the enzyme assay; at 32°C and at pH 6.0, the ATPase activity was found several times greater than at 12°C at pH 6.8. But it is to be noted that a similar situation exists in rabbit actomyosin, though it may not be expected in pecten actomyosin which functions at much lower temperatures (about 5°C).

In insect indirect muscle, the efficiency of the ATP produced especially in sarcosomes through oxidative phosphorylation (Sacktor, '54; Lewis and Slator, '54) to the contractile protein may be better than in muscles of the other animals (cf. Watanabe and Williams, '53). The honeybee cannot fly out for the first several days after emergence, although the ATPase activity of muscle homogenate in the presence of Ca ions changes little during the early adult life, while the Mg-activated considerably increases (Sakagami, Sh. F. and K. Maruyama, unpublished). However, these facts may not deny the prime role of actomyosin ATPase in muscular contraction: the actomyo-

sin ATPase activity has been observed to change in quite parallel with the *actual* muscular function during imaginal differentiation of the wasp, *Vespula lewisii* (Maruyama, '54a) and of the housefly (Maruyama, '54b).

Taking the present study into consideration, the ATP-actomyosin system can be considered to be responsible for muscular function at the molecular level in insects as well as in vertebrates.

ACKNOWLEDGMENT

I wish to express my gratitude to the Director and Staff of the Yamashita Biochemical Laboratory, Hokkaido University for placing much of the laboratory facilities at my disposal. Especially I am indebted to Dr. Y. Tonomura for his kind invitation and for his helpful advice and also to Dr. K. Yagi for his very skillful assistance in light-scattering and viscosimetry studies. Acknowledgments are due to Dr. Sh. F. Sakagami for his generous supply of the material. Finally I take pleasure in thanking Dr. J. Ishida (Tokyo University) for his warm encouragment.

SUMMARY

Some physiocochemical observations were made on the interaction between actomyosin from honeybee thoracic muscle and adenosine triphosphate (ATP). ATP caused superprecipitation, viscosity drop, and light-scattering drop in insect actomyosin. On the other hand, actomyosin showed a powerful ATP-splitting enzyme action. The extent of these interactions of insect actomyosin with ATP was fairly comparable with those of rabbit actomyosin. From the viewpoint of comparative biochemistry, these observations, in general, support the current theory that the ATP-actomyosin system is essential for muscular contraction.

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# THE ROLE OF THE CORIUM IN ION TRANSPORT ACROSS THE ISOLATED FROG SKIN [1]

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TWO FIGURES

Since the pioneer work of du Bois Reymond ('57), the origin of the potential across the isolated frog skin, and the anatomical location of the active site, or sites, has been a subject of considerable debate among physiologists. The predominant, but by no means unanimous, opinion has been that the potential arises in the epidermis. As the subject has been reviewed recently (Ottosen et al. '53), only a few papers will be cited here.

Since the clear demonstration of Ussing ('48) that the isolated frog skin is able to transport sodium ion actively, and his proposal that the potential arises as a result of the active transport (Ussing '48, 49, Ussing and Zerahn '51), the search for the active site gained new impetus. Ussing suggested that the active site probably lay in the basal cell layer of the *stratum germinativum.* In Ussing's view, the corium, being largely muscle fibers and connective tissue, is too diffuse a structure to act as a barrier to diffusion (Ussing '48).

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The question was apparently settled when Ottosen et al. ('53) reported the presence of a thin membrane lying just below the *stratum germinativum,* and the finding that the potential disappeared when the membrane was pierced by a microelectrode. Ottosen et al. ('53) considered that the major portion of the potential was probably located across this newly discovered membrane. Linderholm ('54) suggested that the membrane was the site of active transport.

There are, however, a number of reports in the literature which suggest that the corium might play some role in regulating the permeability properties of the skin. Steinbach ('33) measured the potential from the skin to both solutions as well as from solution to solution. Potassium chloride lowered the potential between the skin and both solutions regardless of the side to which it was added. Calcium ion, on the other hand, lowered the potential between the skin and both solutions when applied outside, but not when applied inside. Further, the potential was found to be remarkedly insensitive to changes of electrolyte concentration inside, but showed large changes when the concentration of the outside solution was changed. Takenaka ('37) studied the lithium oscillation of the potential and found that the oscillation was diminished or abolished by chloroform, the best results being obtained when it was added outside. Ethyl ether or phenyl urethane were more effective if added inside. Barnes ('40) reported that acetylcholine increased the potential when applied outside and depressed it when added inside. Kirchner ('55) found that atropine or d-tubocurarine added outside increased the current across the short-circuited skin. If added inside, these reagents caused only a transient increase. Kato et al. ('56) report that A. T. P. increases the potential if added outside, and decreases it if added inside. Histamine decreases the potential if added to the inside solution, but is ineffective if added outside.

These observations all suggest that the corium does regulate the permeability of the skin to some substances at least, and might be important in maintaining the potential across the skin.

An examination of a cross-section of abdominal skin (Fig. 1A) reveals a thin layer, two or three cells thick, lying basal to the *stratum compactum*. It has previously been referred to as the basal cell layer (Fleming '55), but should, perhaps, be called the *tela subcutanea* (Gaupp, cited by Dawson '20), a tissue layer which is continuous with the subcutaneous tissues. If so, it becomes a matter of definition as to whether or not it should be considered a part of the corium. In any case, the

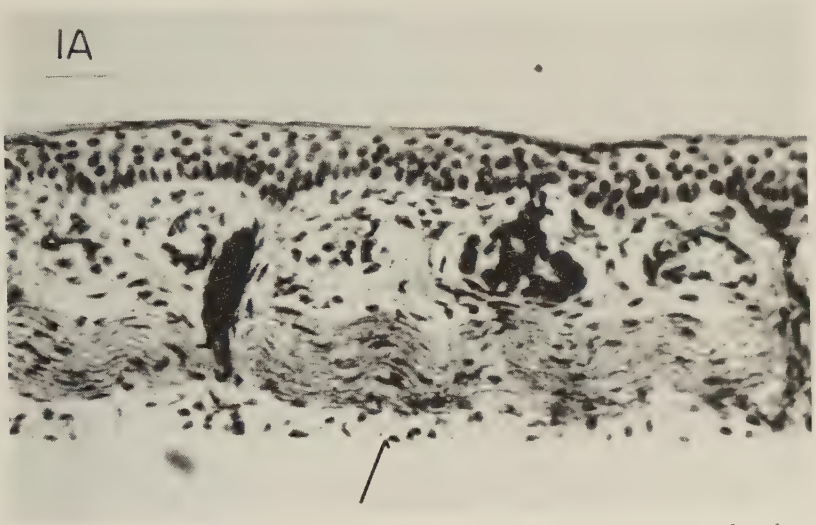


Fig. 1A Cross-section through adbominal skin. Arrow indicates basal cell layer.

removal of this layer offers a possibility of testing the role of the corium in maintaining the potential across the skin, and its role, if any, in the active transport of sodium ion. If the corium is largely an inert tissue, and if the potential is located across the membrane discovered by Ottosen et al. ('53), the removal of this layer should not have much effect upon the bioelectrical properties of the skin.

## METHODS

The studies were carried out using carefully matched plexiglass chambers of the type described in a previous com-

munication (Fleming '57). A frog was killed by decapitation, the spinal cord destroyed, and the abdominal skin removed. By using large females, and cutting up laterally into the pigmented area, it was possible to obtain enough skin to supply both chambers. The potentials taken from two adjacent areas of abdominal skin usually match within 5%. The isolated skin was divided into two equal portions and the basal cell layer removed from one portion, using sharp jeweler's forceps. With some practice the basal cell layer can be removed in a few minutes. The results of such an operation are shown in


Fig. 1B Basal cell layer removed.

Figure 1B. Influx and outflux studies were carried out in the following manner: The operated skin and control skin were mounted in the cells and the potentials recorded. After thirty minutes equilibration, $Na^{22}$ was added and flux measurements made for two one hour periods. Influx and outflux measurements were carried out in parallel, a control skin being run in every case.

At first, all attempts to measure sodium influx and outflux on two operated disks from the same animal failed, the potentials being so low that any differences made it impossible to decide whether or not transport was taking place. It was found, however, that if the basal cell layer was stripped from

the isolated skin, and that skin left to equlibrate for several hours in ringer, the potentials were large enough, and matched closely enough, to make such comparisons possible. A positive deviation from Ussing's ('49b) diffusion equation was accepted as evidence that transport was occurring.

The experimental solution contained 50mM NaCl and 5mM KCl per liter and was titrated to pH 7.5 immediately before use. The counting procedure has been described (Fleming '57). The counting error was kept within 3%.

## RESULTS

As shown in table 1, removal of the basal cell layer reduces the potential considerably.

Along with the reduced potential, an increase in sodium influx is also observed. Representative data is shown in table 2. In 22 skins tested, the average increase over the controls was 59%, with a range of 0% (one flux period) to 158%.

Removal of the basal cell layer greatly increases sodium outflux as shown in table 3. In the 17 skins tested, the average increase over the controls was 469%, with a range of 140% to 1220%.

Finally, a reduced flux ratio is observed. As shown in table 4, the flux ratio obtained from operated skins is usually slightly lower than that predicted from Ussing's (496) diffusion equation.

TABLE 1

*Potentials, in millivolts, of normal frog skin compared with an operated portion of the same skin (basal cell layer removed)*

| FROG | CONTROL | OPERATED |
|---|---|---|
| 1 | 35 | 8 |
| 2 | 17 | 7 |
| 3 | 9 | 4 |
| 4 | 43 | 7 |
| 5 | 22 | 4 |
| 6 | 7 | 1 |
| 7 | 19 | 6 |
| 8 | 51 | 10 |

TABLE 2

*$Na^{22}$ influx ($\mu$ eq./cm²/hr.) and potentials (millivolts) across normal frog skin compared with an operated portion of the same skin*

Observations are for successive one-hour periods

| FROG | | INFLUX | p.d. | INFLUX | p.d. |
|---|---|---|---|---|---|
| 1 | Control | 0.75 | 15 | 0.90 | 20 |
| | Operated | 0.85 | 9 | 0.90 | 14 |
| 2 | Control | 0.76 | 22 | 0.88 | 23 |
| | Operated | 1.10 | 8 | 1.08 | 11 |
| 3 | Control | 0.45 | 18 | 0.61 | 21 |
| | Operated | 0.99 | 4 | 1.03 | 7 |
| 4 | Control | 0.44 | 25 | 0.69 | 26 |
| | Operated | 0.72 | 9 | 0.89 | 12 |
| 5 | Control | 0.66 | 22 | 0.63 | 21 |
| | Operated | 1.12 | 7 | 1.60 | 8 |

TABLE 3

*$Na^{22}$ outflux ($\mu$ eq./cm²/hr.) and potentials (millivolts) across normal frog skin compared with an operated portion of the same skin*

Observations are for successive one-hour periods

| FROG | | OUTFLUX | p.d. | OUTFLUX | p.d. |
|---|---|---|---|---|---|
| 1 | Control | 0.14 | 32 | 0.21 | 39 |
| | Operated | 0.34 | 13 | 0.60 | 15 |
| 2 | Control | 0.06 | 15 | 0.12 | 19 |
| | Operated | 0.42 | 4 | 0.66 | 5 |
| 3 | Control | 0.05 | 26 | 0.08 | 28 |
| | Operated | 0.21 | 11 | 0.37 | 13 |
| 4 | Control | 0.03 | 18 | 0.02 | 21 |
| | Operated | 0.26 | 3 | 0.25 | 7 |
| 5 | Control | 0.07 | 31 | 0.05 | 33 |
| | Operated | 0.22 | 7 | 0.31 | 13 |

TABLE 4

*Influx and outflux of $Na^{22}$ ($\mu$ eq./cm²/hr.) and potentials (millivolts) across two disks of operated skin taken from the same animal*

$M_1/M_2$ calc. is calculated using the average potential of each matched set, using Ussing's diffusion equation. $M_1/M_2$ exp. are the observed flux ratios

| FROG | INFLUX | p.d. | OUTFLUX | p.d. | $M_1/M_2$ CALC. | $M_1/M_2$ EXP. |
|---|---|---|---|---|---|---|
| 1 | 0.80 | 15 | 0.44 | 16 | 1.8/1 | 1.8/1 |
| 2 | 1.10 | 18 | 0.55 | 18 | 2.0/1 | 2.0/1 |
| 3 | 0.94 | 25 | 0.38 | 26.5 | 2.8/1 | 2.5/1 |
| 4 | 0.80 | 28 | 0.46 | 27.5 | 3.0/1 | 1.7/1 |
| 5 | 0.53 | 25 | 0.25 | 23 | 2.6/1 | 2.1/1 |
| 6 | 0.43 | 26 | 0.28 | 26.5 | 2.8/1 | 1.5/1 |

## DISCUSSION

As shown in the data above, the removal of the basal cell layer alters the permeability properties of the skin drastically. One observes a greatly increased influx and outflux of sodium (tables 2 and 3), a reduction of sodium flux ratios to those predicted from diffusion alone (table 4), and the loss of the major portion of the potential (tables 1, 2, and 3).

The reason why the potential is reduced so drastically remains, at this point, obscure. There is, of course, the possibility that the thin membrane described by Ottosen et al. ('53) has been destroyed, but this is believed to be doubtful. If the structure referred to here as the basal cell layer is the *tela subcutanea* of Gaupp (Davenport '20), a cell layer which is continuous with the subcutaneous tissues, it is difficult to believe that it also connects with the membrane of Ottosen et al. ('53).

Actually, the question seems critical. If one supposed that the thin membrane is destroyed, the results obtained by removal of the basal cell layer are easily explainable. According to Ottosen ('53), the potential arises across the newly discovered membrane. If, as Ussing ('48, '49, '51) maintains, the potential arises as the result of the active transport of sodium ion, one must conclude with Linderholm ('54) that the membrane is the site of active transport. Thus one would expect sodium transport, and the potential, to disappear upon rupture of the membrane.

Such a supposition would seem to require that the thin membrane should be considered a double membrane, one part being located below the *stratum germinativum* and being connected to the other portion which lies basal to the *stratum compactum*. Such a possibility cannot be ruled out until further anatomical studies have been carried out.

A simpler assumption, perhaps, would be that the basal cell layer is not continuous with the thin membrane of Ottosen et al. ('53), but that it is also important in regulating the permeability properties of the skin and in maintaining the functional integrity of the tissue. In confirmation of this view,

Crisera and Scheer (Personal Communication) have recently found that the potential drop across the skin occurs in two steps, one being localized just below the epidermis, the other deep in the corium near the basal cell layer. Further, Koblick ('57) has recently studied the cholinesterage activity of frog skin, and finds that over 90% of the enzymatic activity is located in the basal cell layer. Since Kirschner ('53) and Van der Klott ('56) have offered evidence which appears to implicate the chloinesterase system in the transport process, Koblick's ('57) findings, coupled with those reported here, suggest that the cholinesterase system may play an important role in regulating the permeability properties of the skin. It would seem premature, however, to conclude that this is actually the case.

Finally, one must consider the possibility that the data presented here represent nothing more than the results of general mistreatment of the skin. This is believed to be doubtful for several reasons. In the first place, the basal cell layer is easily detached, and with some practice rough handling of the skin can be avoided. Secondly, it has been observed that while rough treatment of the intact skin results in low potentials, such skins will soon recover. Operated skins do not show such a recovery, although the potential will usually increase slowly with time (tables 2 and 3). Finally, methylene blue does not move from the epidermal to the corium side of the skin as would be expected if the skin were punctured or severely damaged (Wertheimer '23).

While further studies are clearly required, the data presented here demonstrate that the corium is an important part of frog skin, and must be intact if the skin is to function properly.

## SUMMARY AND CONCLUSIONS

1. It has been found that the removal of the basal cell layer of the corium has drastic effects upon the physiological properties of the skin. These effects are: (1) a large potential drop, (2) an increased sodium flux in both directions across

the skin, and (3) a reduction of flux ratios to those predicted by Ussing's diffusion equation.

2. It is pointed out that the results obtained require that the skin contain either a double membrane or two separate membranes, both of which must be functional.

3. The data presented here, coupled with that of other laboratories, suggest that the cholinesterase system may be involved in the active sodium transport process.

ACKNOWLEDGMENT

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# ACTIVE CATION TRANSPORT IN SUBMERGED AQUATIC PLANTS

## I. EFFECT OF LIGHT UPON THE ABSORPTION AND EXCRETION OF CALCIUM BY POTAMOGETON CRISPUS (L) LEAVES

BENJAMIN LOWENHAUPT [1]
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FOUR FIGURES

### INTRODUCTION

Active transport [2] in submerged aquatic plants has been little studied. This is unfortunate because several aspects of their structure and physiology are nearly made to order for transport studies. A series of experiments has been conducted to test effects of light under several conditions.

Previous studies of cation transport in aquatics have all been in conjunction with photosynthesis studies. Two names are notable in this connection, Arens and Steemann Nielsen. Arens ('30) ('33) ('36a) ('36b) began his investigations to ascertain why many aquatic leaves become encrusted with lime on the adaxial surface. He demonstrated that leaves that become thus encrusted can photosynthesize bicarbonate (in addition to carbon dioxide) from the external medium and that movement of cations through the leaf from the abaxial (morphological lower) to the adaxial (morphological upper) side is associated with this photosynthesis. He found precise equivalence between the bicarbonate assimilated at the abaxial surface and the cations transported. Steemann Nielsen ('44, '46, '47, '51, '52) refined Arens' experiments so that there can now be no question of the reality of cation transport

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[2] *Transport* or *active transport* refers to the metabolic pumping of material across an active membrane.

or of the stoichiometry between bicarbonate assimilation at the abaxial surface and transport. Ruttner ('53) has reviewed the evidence for bicarbonate assimilation in submerged aquatics, and Österlind ('49) has summarized the history of the study of photosynthesis in aquatics.

The relationship between photosynthetic assimilation of bicarbonate and cation transport affords little doubt that light is linked to transport through photosynthesis. All previous efforts to explain this link have invoked an anion pump, whereas cations were assumed to be carried electrochemically. The emphasis on anions probably was the result of the experimental emphasis on photosynthesis. This made observations of cation movement seem secondary to uptake of the bicarbonate anion. I have rejected this view, however, partly because there is no evidence for anion transport through the leaf. Instead I have suggested that there is cation transport, powered by photosynthesis, and that associated biochemical reactions entail a carboxylation that uses bicarbonate ions. It is impossible to present this theory fully here, but it has been considered in another paper (Lowenhaupt, '56). The data presented here are some of the observations from which the theory was inferred.

## MATERIAL AND METHODS

*Potamogeton crispus* plants were grown in an artificial pond. For each experiment healthy-looking, full-grown leaves were brought into the laboratory.

Calcium-45, as obtained from the Atomic Energy Commission, 15 to 20 mc/g, was purified by precipitating out added zinc and copper as the sulfides and then precipitating the calcium-45 by adding oxalate. The calcium-45 oxalate was dried, weighed, ashed, and dissolved in dilute hydrochloric acid. The excess acid was evaporated and the residue redissolved in water. This yielded a calcium chloride solution approximately neutral and of known concentration.

The experiments required quantitative comparisons for calcium; they were made by radioactivity measurements. This involved placing measured aliquots of solution into a counting

cup, adding water to spread the solution, evaporating the water, and estimating the radioactivity with a Geiger-Muller counter. If radioactivity were proportional to the amount of calcium, i.e., if specific radioactivity did not change, the amount of the element in an unknown sample might be calculated by the ratio of its radioactivity to that of a standard sample.[3] Changes in specific radioactivity did occur, however, because the leaves contained nonradioactive calcium, some of which was excreted during transport while radioactive calcium from the medium was being absorbed. Experience has shown, though, that the specific activity of the external medium becomes constant after a few hours, provided the leaves are illuminated, and thereafter any changes in radioactivity may be considered proportional to changes in calcium content. Evidence for this includes:

(a) the constancy of radioactivity of the medium under uniform conditions, and

(b) the cyclical repetition of radioactivity changes, corresponding with cyclical changes in the environment. Both of these have been observed frequently. Accordingly leaves were pretreated to bring specific radioactivity to constancy throughout the system before experimental procedures were instituted. Radioactivity of the medium, not the leaves, was measured. The radioactivity of the leaves was calculated by substracting radioactivity of the medium from the total radioactivity. (The relative changes in radioactivity of the leaves indicated in the figures are smaller than were the observed relative changes in the media because most of the radiocalcium was in the leaves.)

In the experiments whose results are summarized in figure 1, leaves were floated on an approximately neutral solution of calcium-45 bicarbonate, some leaves abaxial face up, some adaxial face up. Drops of this solution were placed on top of leaves and the dish covered with a watch glass and left in the light for about 1.5 hours. Radioactivity per unit volume of

[3] Autoabsorption can be ignored because the mass of material in the counting cups was small.

the drops on top of the leaves was compared with that of the solution underneath, the latter being taken as 100%. Check experiments were run in the dark with the same leaves after they had been in darkness several hours prior to placing the drops on top.

In the experiments whose results are summarized in figure 2, leaves that had been in darkness for some time were floated with minimal exposure to light on calcium-45 chloride solution (2 ml; $6 \times 10^{-4}$ M), either with all the abaxial faces touching the solution or with all the adaxial faces touching. Thus only one side of any set of leaves had the potentiality of transport, the other side being dry. Darkness was continued for about 2 hours and after this the periods of light and darkness were alternated, as indicated in figure 2. At intervals throughout the experiment radioactivity of the medium was determined. Occasionally leaves were turned over, as indicated in the figure.

In the experiment whose results are summarized in figure 3 leafy shoots, from which the growing tip and attached immature leaves had been removed, were submerged in 10 ml of calcium-45 chloride solution ($10^{-4}$ M). The vessel was covered with a watch glass and the whole illuminated. At approximately 2-hour intervals the solution was replaced by 10 ml of fresh radiocalcium solution to load the leaves with radioactivity. After three applications they were left illuminated overnight. The next day, periods of darkness and illumination were alternated as indicated in figure 3. Changes in radioactivity of the medium were followed throughout.

The results indicated in figure 4 were obtained in an experiment similar to that of figure 3. It differed, however, in that during the second dark cycle, at the time indicated, hydrogen peroxide ($5 \times 10^{-2}$ millimols total; concentration in medium $5 \times 10^{-3}$ M) was added to the medium.

## RESULTS

The results when drops of $Ca^{45}(HCO_3)_2$ solution were laid on top of floated leaves are summarized in figure 1. Calcium

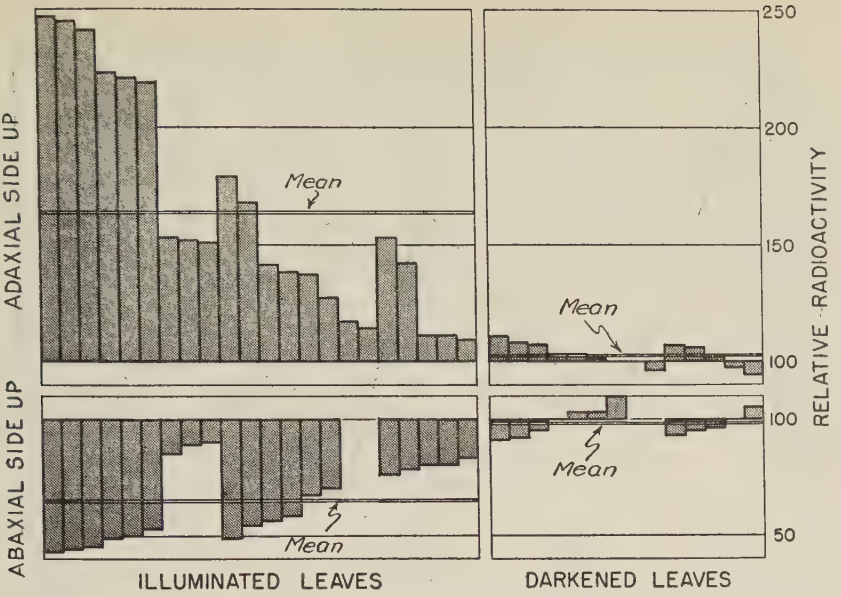


Fig. 1 Effect of light vs darkness on the relative calcium concentration of drops of $Ca^{45}(HCO_3)_2$ solution on top of floated leaves, as measured by radioactivity determinations.

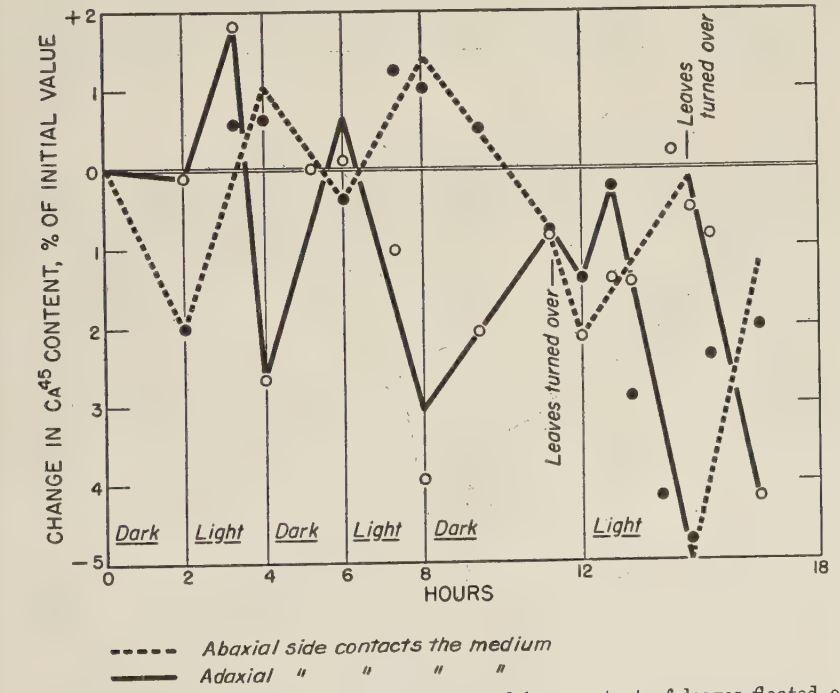


Fig. 2 Effect of light vs darkness on the calcium content of leaves floated on $Ca^{45}Cl_2$ solution, as indicated by radioactivity determinations.

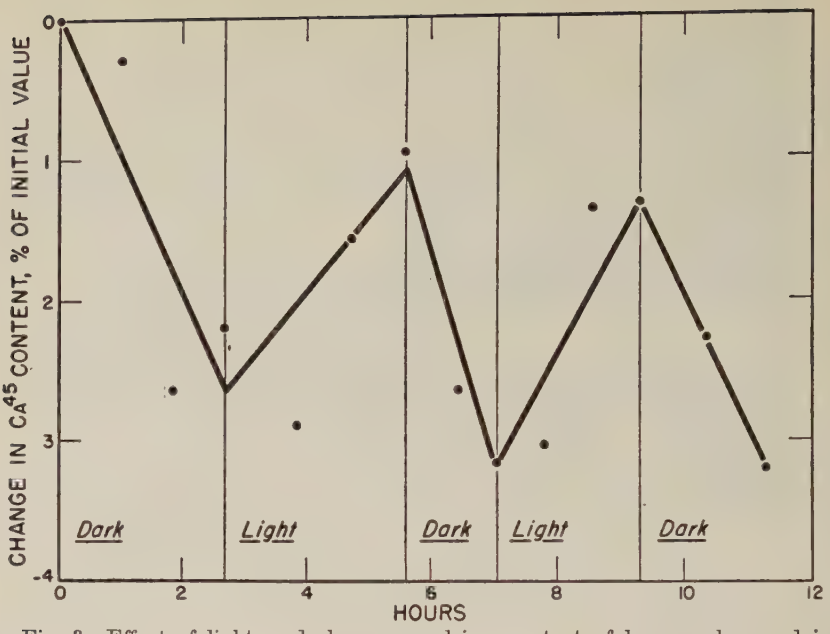


Fig. 3 Effect of light vs darkness on calcium content of leaves submerged in $Ca^{45}Cl_2$ solution, $10^{-4}$ M, as indicated by radioactivity determinations.

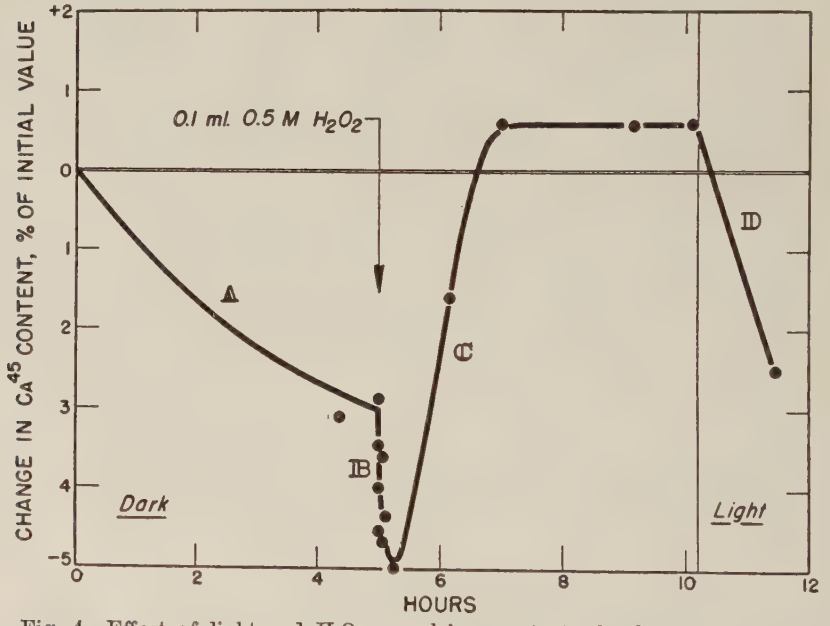


Fig. 4 Effect of light and $H_2O_2$ on calcium content of submerged leaves, as indicated by radioactivity determinations. (Experimental conditions were similar to those of figure 3 except that $H_2O_2$ was used).

concentration is lowered in the solution that lies on the abaxial faces of illuminated leaves and is raised in the solution that lies on the adaxial faces. In darkness the solutions on the two surfaces of the leaves do not differ significantly from each other in concentration.

Figure 2 indicates the absorption and excretion of calcium by floated leaves. In the light, calcium moved from the medium into the abaxial leaf faces, and it moved out of the adaxial faces. In the dark, on the other hand, calcium movement tended in the opposite directions.

Effects of alternating illumination and darkness on submerged leaves are indicated in figure 3. In the light leaves held more calcium than in the dark.

Results of the experiment shown in figure 4 were similar to those of figure 3 until the application of hydrogen peroxide (A). Then calcium moved out of the leaves for about half an hour (B). After that it moved into the leaves (C). Illumination then caused calcium to move out of the leaves, into the medium, as indicated at D.

## DISCUSSION

Arens (loc. cit.) and Steemann Nielsen (loc. cit.) have previously observed the movement of cations through *Potamogeton* and similar leaves. Results shown in figure 1, obtained with calcium–45, substantiate several important conclusions reached by Arens and Steemann Nielsen, using different methods, for a number of cations.

From the results shown in figure 1 it may be concluded that:

(a) Calcium is accumulated into the abaxial leaf face in the light from its contiguous medium and is excreted from the adaxial face;

(b) In darkness, calcium movement does not occur.

Of the results shown in figure 2, those for the light are consistent with the above conclusions. There is a contrast, however, between the dark results of figures 1 and 2; in the former case (fig. 1) calcium movement in the dark did not occur, whereas in the latter the movement reversed the direction in

the light. This difference is explained by the experimental differences. The dark results presented in figure 1 were obtained for leaves from which light was excluded, both for some time before the experiment and during it. But the results for figure 2 were obtained by alternating light and dark periods.

One concludes from the latter results that darkness creates no inhibitor for the reactions of transport, but rather that transport stops in the dark for lack of power. As a result (one infers), the reactions of transport all assume their equilibrium levels. This interpretation is consistent with the dark results of figure 1 because in those experiments equilibrium would have been attained during the pretreatment in the dark.

The results summarized in figure 3 contrast the equilibrium level in the dark with the steady-state level of calcium in illuminated leaves. Notable in this experiment is the shift back and forth from steady state to equilibrium when the light is turned off and on. The exact levels for these have, however, been found to depend upon experimental conditions, so that the size of this shift and even its direction (i.e. whether the leaves in the dark hold more or less calcium than illuminated leaves) can be controlled by the treatment.

The results shown in figure 4 demonstrate that treatment may change the equilibrium (dark) level of calcium. The change from light to darkness shifted the calcium content from the steady-state level to the equilibrium level, about as it did in the experiment of figure 3. But adding hydrogen peroxide introduced another factor. The immediate effect was to release calcium. In another report (page 209, this journal) is evidence that oxygen is necessary for the chemical reactions releasing calcium from the leaves. The initial effect of hydrogen peroxide is consistent with this suggestion. But the leaves decompose hydrogen peroxide. Apparently when the hydrogen peroxide was gone, in the experiment of figure 4, a new equilibrium was established in which more of the calcium was contained in the leaves. It would seem, therefore, that calcium release from the leaves employs a biochemical

material labile in the presence of hydrogen peroxide. The ability of light to release calcium from leaves injured by hydrogen peroxide would indicate, further, that photosynthesis powers calcium transport by means of this labile, calcium-release material.

## SUMMARY AND CONCLUSIONS

In experiments on the relation between illumination and calcium transport in *Potamogeton crispus* leaves, the following observations were made:

1. Calcium was accumulated into the abaxial surface of illuminated leaves and was excreted from the adaxial surface.
2. Calcium transport did not occur in the dark.
3. The calcium content of illuminated leaves differed from that of darkened leaves.
4. Exposure to hydrogen peroxide increased the calcium-holding capacity of darkened leaves.
5. Light then released calcium from these leaves.

The following explanations are suggested for these observations:

1. Light powers calcium transport in *Potamogeton crispus* leaves.
2. The calcium content of illuminated leaves assumes a steady-state level that is determined by the rates of the reactions for calcium entry into the leaves and release therefrom.
3. The calcium content of darkened leaves is determined by the equilibria of the reactions of transport.
4. There is a material, labile in the presence of hydrogen peroxide, that reduces the equilibrium level of calcium in darkened leaves.
5. Light synthesizes this material.

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## ACKNOWLEDGMENT

Heartfelt thanks are tendered Professors J. P. Bennett, Roy Overstreet, and M. A. Joslyn for their generous advice and encouragement.

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# ACTIVE CATION TRANSPORT IN SUBMERGED AQUATIC PLANTS

## II. EFFECT OF AERATION UPON THE EQUILIBRIUM CONTENT OF CALCIUM IN POTAMOGETON CRISPUS (L) LEAVES [1]

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THREE FIGURES

### INTRODUCTION

Cation transport can be observed in the leaves of submerged aquatic seed plants more advantageously than in other biological materials. A series of experiments has been undertaken to exploit the advantages of these leaves. Two reports of this series have preceded this one. The first of these (Lowenhaupt, '56) surveys cation transport with special reference to its mechanism in submerged aquatic plants. The following generalizations are developed for most submerged aquatic seed plants:

1. Cations journey through the leaf in the light; they are pumped twice en route. The active membrane on the abaxial surface transports them from the contiguous external medium into the leaf cells in the light while the active membrane on the adaxial leaf surface transports them out of the leaf into its contiguous external medium.
2. These two active membranes consist of the cell plasma membranes on the abaxial and adaxial leaf surfaces respectively.
3. Action at the active membranes involves three steps in the light:
   a. cation attachment to a binding group,
   b. reorientation of the group toward the other surface of the active membrane,
   c. release of the cation.

   The energy needed for transport is provided by photosynthesis. Direct application of this energy is for the cation-release step.

[1] This work supported in part by the U.S. Atomic Energy Commission.

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4. In darkness, the cation transport mechanism is shut off. The biochemical constituents of this mechanism then reach resting levels. These levels are functions of the equilibria of the reactions between different constituents; therefore, one can alter the resting level of one constituent by controlling the supply of another.
5. In the light, when the transport mechanism is functioning, the levels of the biochemical constituents reach steady-state values. These levels, generally different from the resting levels, are functions of the rates of the reactions between the constituents. Therefore, one can alter the steady-state levels by suitably altering the conditions of one or more of these reactions.
6. The transport mechanism at the active membrane supplies links of a respiratory chain. Cation attachment is an oxidation-reduction reaction. Organic reserves implement the action of a reducing agent that reduces the carrier in the active membrane. As a result the carrier attaches a cation. Cation release is likewise an oxidation-reduction reaction. An oxidizing agent formed as a result of photosynthesis liberates the cation from the carrier.
7. The transport reactions synthesize a bicarbonate-accepting compound at the plasma membrane. Apparently, carbon from external bicarbonate ions is thus enabled to penetrate the plasma membrane and so reach the site of photosynthesis inside the cells.

The second report (Lowenhaupt, '58) presents data relating illumination to calcium transport through *Potamogeton crispus* leaves. The following conclusions are drawn:

1. Light powers calcium transport in *Potamogeton crispus* leaves.
2. The calcium content of illuminated leaves assumes a steady-state level that is determined by the rates of the reactions for calcium entry into the leaves and release therefrom.
3. The calcium content of darkened leaves is determined by the equilibria of the reactions of transport.
4. There is a material, labile in the presence of hydrogen peroxide, that decreases the equilibrium level of calcium in darkened leaves.
5. Light synthesizes this material.

This report presents data on the effects of aeration upon the equilibria in the *Potamogeton crispus* leaf for the reactions

of calcium accumulation and excretion. The results reported here are some of the bases for generalization 6, above.

## MATERIALS AND METHODS

The methods of growing and preparing the *P. crispus* leaves and of preparing and measuring the radio-calcium were described on page 200 of this journal. Three experiments testing effects of aeration upon the amount of calcium in the leaves will be reported.

The first experiment is a modification of the third experiment of the preceding report (Lowenhaupt, '58). It resembles that experiment in that leaves were transferred from illumination to darkness while their calcium content was measured. It differs in that some of the leaves were supplied with nitrogen instead of air when the light was first off. A variation of this experiment was also run in which air was supplied to leaves during one dark period. Then, after a period in the light, the leaves were again put in the dark. This time nitrogen was supplied for the first hour and air thereafter.

In the second experiment leaves were washed in HCl solution (pH 3.5) and transferred to 20 ml of $Ca^{45}Cl_2$ solution ($10^{-3}$ M). (This experiment differs from the others in that leaves were not pretreated with radio-calcium solution. Accordingly, changes in radioactivity cannot be considered proportional to changes in calcium content.) After an hour's illumination leaves and medium were divided into two equal portions, both of which were placed in the dark. Eight $\times$ $10^{-3}$ millimoles of $K_2CO_3$ were added to each portion and in one the air was replaced with nitrogen. The other was left undisturbed. After an hour air was again supplied to the anoxic portion. Radioactivities and pH's of both media were followed throughout the experiment and changes in the radioactivity of the leaves were calculated from the observed radioactivities in the medium.

In the third experiment, leaf segments about five mm long were used. After several hours immersion in $Ca^{45}Cl_2$ solution

(initial concentration $10^{-4}$ M) in darkness, these segments were clamped between glass cells filled with the bathing $Ca^{45}Cl_2$ solution. The placing of the leaf segments between the cells was done as quickly and in as dim light as possible. The apparatus was transferred to a metal box and humid air was bubbled into the solution in the upper cell through a

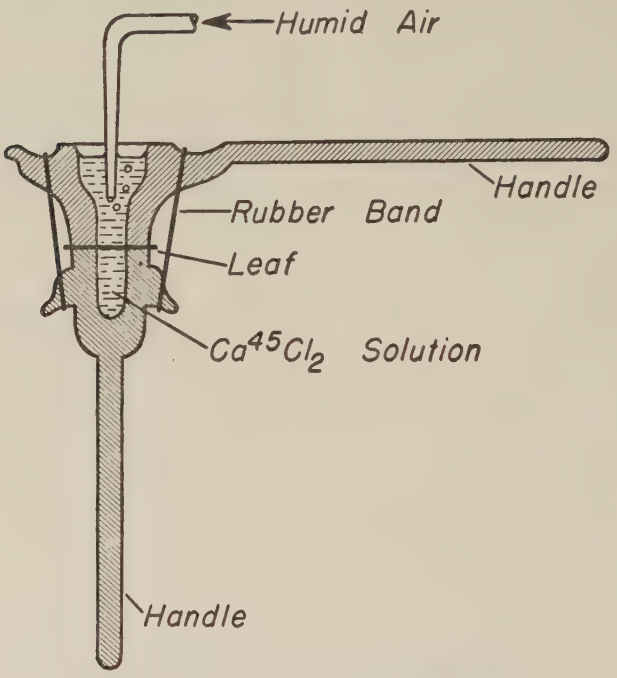
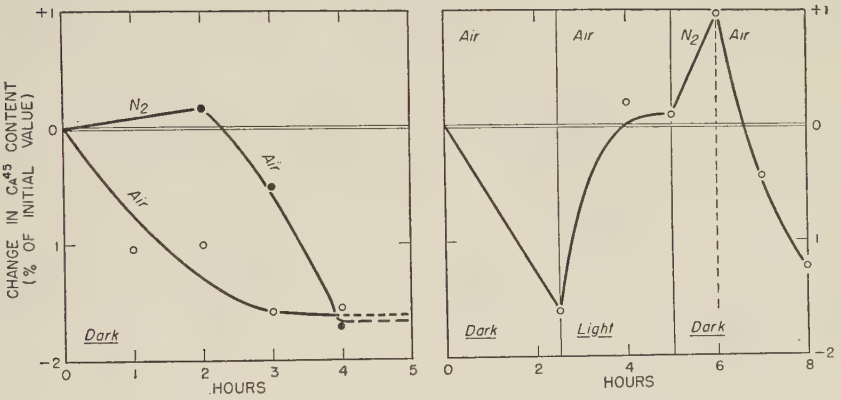



Fig. 1 Diagram of the set up for the third experiment. (Scale: 1½ times actual dimensions.)

capillary tube; the solution in the lower cell remained unaerated. Figure 1 diagrams the experimental setup inside the box. The inner walls of the box were sprayed with water and the box closed. Air was introduced in the dark for about six hours. Again avoiding light and working as quickly as possible, the two cells were separated. The radioactivities of measured aliquots from each were compared with that of the $Ca^{45}Cl_2$ solution before the experiment, the latter value being taken as 100%.

## RESULTS

Leaves transferred from light to darkness gave up part of their calcium to the medium and regained it when illuminated. (Figures 2a and 2b.) This result is similar to those reported in the third experiment of the preceding report. When nitrogen, instead of air, was supplied to the darkened leaves, however, a different result was obtained. Then, instead of re-




Figs. 2a and 2b Effect of air vs. nitrogen on calcium content of leaves submerged in $Ca^{45}Cl_2$ solution, $10^{-4}$ M, as indicated by radioactivity determinations (leaves transferred from light to darkness at the beginning of both experiments).

Fig. 2a (left) contrasts the calcium content of two lots of leaves, one supplied with air throughout the experiment, the other supplied with nitrogen for two hours.

Fig. 2b (right) shows effects of a succession of treatments on a single lot of leaves.

leasing calcium, the leaves accumulated it from the medium. When air was restored, calcium was released.

Leaves that had been in HCl solution removed about 40% of the radioactivity from a $Ca^{45}Cl_2$ solution within one hour and the acidity of this solution increased from a pH value slightly less than 7.0 to pH 3.8 (fig. 3). When $K_2CO_3$ was added to counteract the acidity, the medium first became alkaline, then returned gradually to pH 5.8. Radioactivity entered both experimental groups of leaves after the $K_2CO_3$ was added, but it entered more rapidly and completely into those deprived of oxygen. As a result, the anoxic leaves con-

tained about 400 counts per second more radioactivity after an hour than the leaves in air. When air was restored to the oxygen-deficient leaves, about 150 counts per second of radioactivity moved out in about one and one-half hours. During the same time radioactivity continued to enter the other set

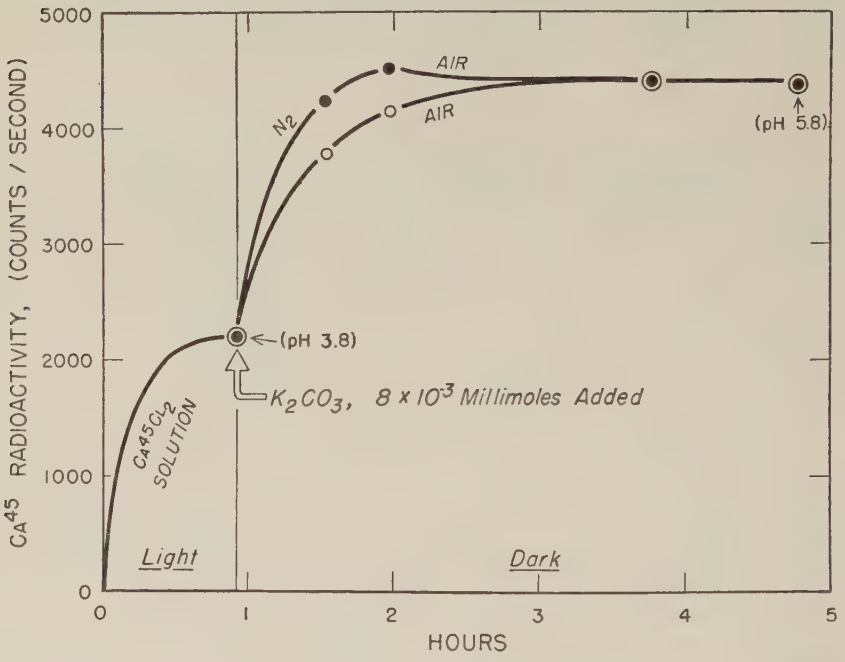



Fig. 3 Effect of aeration on calcium-45 radioactivity uptake by leaves. (These leaves, washed first in HCl solution, pH 3.5, and then in water, were placed in $10^{-3}$ M $Ca^{45}Cl_2$ solution, the pH of which fell from about pH7 to pH3.8.)

of leaves that had been in air throughout so that final radioactivities of the two were the same.

There was a shift of calcium across leaves aerated from one side (table 1). This is in contrast to the unchanging calcium concentration on the two sides of dialysis tubing or aluminum foil (table 2). The shift when a leaf was used was toward the aerated medium regardless of whether this was in the abaxial to adaxial direction or vice versa. The amount of calcium removed from the medium on the adaxial side of the leaf is

small (4% of the initial calcium in the medium; it is significant, however). This smallness results from the difficulty of controlling conditions during an experiment of several hours so that there is no net loss or gain of calcium from the leaves. Small relative changes in the leaf calcium cause large changes in the medium because the calcium concentration in the leaves is greater than in the medium. Actually, however, the difference in calcium content between the aerated and unaerated media is more important than the absolute gain or loss from either medium.

TABLE 1

*Effect of unilateral aeration on relative calcium content of medium on either leaf face as indicated by radioactivity determinations*

Leaves that had been in darkness overnight were kept in the dark during the experiment. Medium at the outset on both leaf faces was $Ca^{45}Cl_2$ solution, $10^{-4}$ M, having a relative radioactivity of 100%

| LEAF ORIENTATION | A RELATIVE RADIOACTIVITY AERATED SOLUTION | B RELATIVE RADIOACTIVITY UNAERATED SOLUTION | DIFFERENCE (A — B) |
|---|---|---|---|
| | % | % | % |
| | 127 | 99 | 28 |
| Adaxial | 121 | 107 | 14 |
| | 107 | 94 | 13 |
| Side | 116 | 90 | 26 |
| | 106 | 89 | 17 |
| Aerated | 107 | 97 | 10 |
| | 120 | 87 | 33 |
| | 108 | 106 | 2 |
| | Mean = 114.0 | Mean = 96.1 | Mean = 17.9 |
| | Range = 106–127 | Range = 87–107 | Range = 2–33 |
| Abaxial | 136 | 80 | 56 |
| | 115 | 88 | 27 |
| Side | 104 | 99 | 5 |
| | 94 | 59 | 35 |
| Aerated | 107 | 96 | 11 |
| | Mean = 111.2 | Mean = 84.4 | Mean = 26.8 |
| | Range = 94–136 | Range = 59–99 | Range = 5–56 |
| | Mean of all = 112.9 | Mean of all = 91.6 | Mean of all = 21.3 |
| | Range of all = 94–136 | Range of all = 59–107 | Range of all = 2–56 |

(Statistical analysis indicates significance of all differences: A — B; A — 100%, and 100% — B for both leaf orientations. Thanks are due Professor Lucien Le Cam for this analysis.)

TABLE 2

*Effect of unilateral aeration on relative calcium content of medium on either face of inert membranes in the dark as indicated by radioactivity determinations*

Experimental conditions were similar to those of table 1 except that inert membrane material was used instead of leaf

| MEMBRANE MATERIAL | A RELATIVE RADIOACTIVITY AERATED SOLUTION | B RELATIVE RADIOACTIVITY UNAERATED SOLUTION | DIFFERENCE (A — B) |
|---|---|---|---|
| | % | % | % |
| | 101 | 97 | 4 |
| | 105 | 105 | 0 |
| Dialysis | 104 | 104 | 0 |
| | 106 | 102 | 4 |
| | 97 | 101 | —4 |
| | 100 | 102 | —2 |
| Tubing | 96 | 97 | —1 |
| | 104 | 97 | 7 |
| | Mean = 101.6 | Mean = 100.6 | Mean = + 1.0 |
| | Range = 96–106 | Range = 97–105 | Range = —4 – + 7 |
| | 106 | 103 | 3 |
| | 101 | 99 | 2 |
| Aluminum | 102 | 107 | —5 |
| | 101 | 100 | 1 |
| | 104 | 99 | 5 |
| Foil | 102 | 96 | 6 |
| | 99 | 94 | 5 |
| | — | 107 | — |
| | Mean = 102.1 | Mean = 100.6 | Mean = + 2.4 |
| | Range = 99–106 | Range = 94–107 | Range = —5 – + 6 |
| | Mean of all = 101.9 | Mean of all = 100.6 | Mean of all = + 1.7 |
| | Range of all = 96–106 | Range = 94–107 | Range of all = —5 – + 7 |

## DISCUSSION

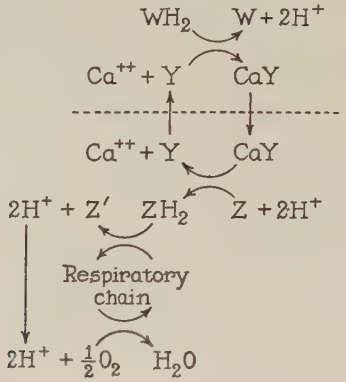
Oxygen has long been known to be necessary for active transport in a number of biological systems. These experiments are an attempt to determine where oxygen fits into the transport mechanism. They indicate that oxygen is needed to release calcium from the active membrane.

The first experiment contrasted the equilibrium calcium content of both aerated and unaerated leaves in the dark with

the steady-state content in the light. (That there is equilibrium in the dark and steady-state in the light was demonstrated in the preceding report.) The evidence shows that the equilibrium calcium level is reduced by aeration, i.e., that calcium release is implemented thereby. The second experiment utilized the fact that the calcium level in the leaves is a function of the acidity of the medium. The results of this and other experiments indicate that at a pH below 3.8 in the medium, calcium is extracted from the leaves. Neutralization of the acid permits calcium uptake to an equilibrium. The fact that this equilibrium was higher under anoxic conditions indicates that oxygen favors calcium release. The third experiment differs from the other two in that the leaves probably were not injured. Evidence for this is the fact that their net calcium content remained approximately unchanged during the experimental period, whereas injury from anoxia has been observed to cause a gradual release of calcium into the medium. In this experiment, one side of the leaves was aerated and it seems that sufficient oxygen moved through the leaves to maintain the other side. Nevertheless, an oxygen gradient across the leaf must have existed and the shift of calcium into the leaf on the unaerated side and out of the aerated side is evidence that calcium release is favored by oxygen. Although the pH's of the aerated and unaerated media were not measured, it seems unlikely that pH effects could have caused the calcium shift, because the unaerated medium would hardly be more alkaline than the aerated one. One would expect the reverse, if there were any difference, because carbonic acid might accumulate in the unaerated medium whereas it would be swept out of the aerated solution.

One is led to consider how oxygen may function in calcium release. Admittedly a number of speculations can be raised, but most of them seem implausible. The simplest suggestion, and therefore the one to be preferred, is that a respiratory chain links oxygen with a metabolite, part of this chain being the calcium transport mechanism. According to this view, one step in transport occurs when a metabolite reduces the

calcium carrier in the active membrane and as a result, the carrier binds calcium. Oxidation of the carrier, it would seem, must release the calcium on the other side of the active membrane as a subsequent step in transport. These considerations (and others) have led (Lowenhaupt, '58) to the following suggested biochemical mechanism:




where $WH_2$ is the metabolite (in reduced form) that reduces the calcium carrier, W is this metabolite after it has been oxidized, Y is the calcium carrier, Z is the oxidizing agent necessary for calcium release from Y (effects of illumination indicate that Z is formed as a result of photosynthesis), $ZH_2$ is the product of Z formed when Z oxidizes CaY, Z′ is the oxidation product of $ZH_2$, and the dotted line represents the active membrane. The kinship between this mechanism and the redox pump suggested by Conway ('53) is apparent.

## SUMMARY AND CONCLUSIONS

Previous reports have presented:

1. A theory of cation transport in submerged aquatic seed plants and
2. Data supporting parts of this theory.

A summary of the conclusions from these reports is presented in the Introduction of this paper.

This report presents data supporting another aspect of the theory. *Potamogeton crispus* leaves were found to contain,

under suitable conditions, more calcium if anoxic than if aerated. Three methods of demonstrating this phenomenon are presented.

The conclusion is suggested that the reactions of calcium transport are links in a respiratory chain between a metabolite and oxygen. This article was received for publication August 20, 1956.

ACKNOWLEDGMENT

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# ELECTRICAL POTENTIAL MEASUREMENTS ON SINGLE NEPHRONS OF NECTURUS[1]

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TWO FIGURES

Electrical potential differences have been obtained across a variety of actively transporting living membranes. Among the biological membranes involved in the transport of electrolytes the tubular epithelium of the kidney takes an extraordinary position: the flux of most ion species, particularly sodium and chloride, greatly exceeds that of other living membranes such as erythrocytes, muscle, nerve and frog skin (Ussing, '51). The measurement of electrical potential gradients across living membranes has aided considerably in a better understanding of the ion transport mechanisms involved. Thus the distinction between active transport and diffusion is based on observations as to whether ion transfer does occur against an electrochemical gradient or not (Ussing, '54a).

This study is an attempt to measure with microelectrodes the electrical potential difference across the renal tubule wall of single nephrons *in vivo,* using an adaptation of micropuncture techniques. Such measurements have been made by Wilbrandt ('38) using electrolyte-filled quartz electrodes having a tip diameter of about 10 to 15 μ. A small electrical potential difference was observed across the proximal tubule wall of Necturus. However, in using these relatively large electrodes, local damage of the tubule might have been unavoidable and sufficient to produce a significant local shunt, reducing

[1] This study was supported by a grant from the National Heart Institute, National Institutes of Health.

the true potential difference. In this study it was hoped to minimize the possibility of injury at the site of impalement by the use of microelectrodes having a tip diameter of 1 μ or less (Ling and Gerard, '49).

We have also attempted to measure transmembrane potentials of single renal tubule cells and have obtained stable potentials under normal conditions and under circumstances which influence renal tubular electrolyte transport. We have chosen organic mercurial diuretics, compounds which *in vivo* exert a marked effect on the renal tubular transport of sodium and chloride, and *in vitro* inhibit the uptake of potassium into renal tubule cells (Mudge, '51). Ischemia was also used since *in vitro* oxygen lack reduces the maintenance of transcellular ionic concentration gradients in renal cortical tissue slices (Mudge, '51, Whittam, '56).

Our results indicate that an electrical potential difference is maintained across the renal tubular wall of Necturus and that the transmembrane potential of single renal tubule cells is of the order of magnitude found across single muscle fibers, nerves and other excitable membranes (Hodgkin, '51). This potential difference is significantly reduced by oxygen lack and larger amounts of a mercurial diuretic.

## METHODS

*Preparation.* All experiments were performed on adult Necturi, anaesthetized with urethane (Walker and Reisinger, '33), during the months of March to June. Potential measurements were done on animals having vigorous as well as spontaneously occurring poor renal circulation or complete vascular stasis. The latter is not an infrequent occurrence in this preparation. Impalements of single tubules were carried out using a stereoscopic binocular microscope at 60 × magnification and combining a modification of the micropuncture technique decribed by Richards and Walker ('37) with the use of glass microelectrodes (Ling and Gerard, '49). All experiments were done at room temperature which varied between

22 and 27°C. The exact site of tubular impalement was identified in some instances by the neoprene cast procedure of Bott ('52) after electrical recordings had been terminated. During all experiments a layer of amphibian Ringers solution covered the kidney (composition: NaCl 0.60 gm, KCl 0.01 gm, $CaCl_2$ 0.013 gm, $NaHCO_3$ 0.01 gm, water 100.00 gm, pH adjusted to 7.50).

*Micropipette-electrodes.* Microelectrodes were drawn from thin-walled capillary tubing having an outside diameter of 0.8 mm by a capillary-pulling device such as described by Alexander and Nastuk ('53). They were filled by the method of Tasaki et al. ('54) with 3M KCl to which chlorphenolred had been added to give a final concentration of 0.1%. It was necessary to filter the colored KCl solution several times before use. In spite of this, a good deal of trouble was experienced during the ejection of fluid through the electrode (see below) by small dislocations of the electrode tip or clogging. Suitable electrodes had a resistance (measured in Ringers solution) of about 5 to 50 megohms. Breaking a number of electrode tips of each new batch, placed in contact with Ringers solution gave only a small potential change (2–8 mV). Each batch of electrodes was tested in this manner before use and it was hoped thereby to minimize the possibility of using an electrode batch having high junction potentials at the electrode tip (Del Castillo and Katz, '55, Adrian, '56).

During the course of the first experiments the importance of localizing the tip of the recording electrode became evident. From inspection alone we were quite unable to decide with any degree of certainty whether or not the tip of the microelectrode had proceeded into the tubule lumen. Therefore an injection arrangement similar to that described by Grundfest et al. ('54) was adopted. This microinjection apparatus allowed the very slow injection, through the recording microelectrode, of a small amount of 3M KCl which had previously been colored by chlorphenolred. The colorant permits observations of the fluid ejected and, in particular, shows whether the tip of the recording electrode was inside the tubule lumen.

When this is the case, first a distinct filling of the tubule lumen at the site of impalement, and later a sharp outline of the greater part of the nephron is obtained. Thus one is assured of the intratubular location of the recording electrode and, furthermore, can localize the approximate site of impalement along the nephron. We regard the exact location of the electrode tip as essential to an interpretation of the recorded potential since we observed quite frequently no filling of the tubule lumen even when inspection alone strongly suggested the tip of the electrode to be inside the tubule. Under those circumstances the subsequent injection of a small amount of chlorphenolred-colored KCl revealed a small indentation of the tubule wall at the site of impalement. In contrast, cellular impalements were characterized by a minute stain, sharply localized at the electrode tip, the tubule lumen remaining unfilled.

*Recording equipment.* Connection of the microelectrode with the electrical recording apparatus was made through a 125 μ chloride-coated silver wire which extended from the side-arm of the electrode holder through the front end into the lumen of the KCl-filled microelectrode. The indifferent lead was formed by a glass tube (3 mm o.D.) filled with amphibian Ringers solution into which the chlorided silver wire extended. This assembly was mounted on the animal dish in such a way as to form a low-resistance contact with the surface of the kidney. This bridge led to ground via small resistances through which known calibration voltages could be applied.

The recording apparatus consisted of a conventional cathode-follower input stage working into a stabilized carrier-type DC amplifier (Leeds and Northrup, model 5835). Grid current of the cathode-follower input tube (RCA 5693, triode-connected) was of the order of $0.5 \times 10^{-11}$ ampere. Direct current drift of the whole recording system was less than 2 mV/hr after warm-up.

*Pretreatment of animals.* In a number of experiments, chlormerodrin (Neohydrin), tagged with $Hg^{203}$, was injected

subcutaneously prior to the experiments in which the effect of mercurial diuretics was to be tested, in amounts ranging from 2–10 mg Hg/kg. This preparation was chosen because of its ease and convenience of analysis. Thus it was possible to correlate the concentration of the mercurial diuretic in renal tissue with effects observed. We have shown (Giebisch and Dorman, '58) that 24 to 48 hrs. after administration, the mercury uptake by the kidney of Necturus is maximal. Therefore potential measurements were performed after such a time interval. After termination of the experiment the kidney was removed, an appropriate sample of the region in which the potential measurements had been done, weighed, and radioactivity counted in a well-type scintillation counter. Concentration of the diuretic is expressed as micrograms of mercury per gm wet tissue.

*Estimation of Intra-and Extracellular Potassium Concentration.* In 10 animals, intracellular potassium concentration was estimated in the following way: kidneys of anaesthetized Necturi were carefully removed from situ, blotted on filter paper and weighed before and after drying overnight in an oven at 105°C. From the difference between dry and wet weight and assuming an extracellular volume of 26% (Whittam, '56), intracellular water content was calculated. Dry kidney samples were digested in dilute nitric acid on a steam-bath and appropriately diluted samples were compared in a flame photometer with postassium solutions made up in dilute nitric acid. Potassium concentration in extracellular water was measured by collecting blood from the aorta into a heparinized syringe and measuring potassium on plasma samples by flame photometry. A protein content of 2.35% (Walker, Hudson, Findley and Richards, '37) was assumed in calculating the potassium concentration in the extracellular fluid. From values of total and extracellular potassium and water, an estimate of intracellular water and potassium concentration was obtained.

## RESULTS

*Tubular transcellular potentials.* When the microelectrode was lowered upon the kidney it was possible to observe the point of impalement. However, after the electrode had entered the tissue it was impossible to observe the position of the tip with any great degree of accuracy. If dimpling occurred on touching the tissue it was taken as a sign of a blunt tip (Bülbring and Hooton, '54) and quite often only a small potential difference could be obtained. We have selected for cellular impalements mostly the first section of the proximal tubule which can be easily recognized by its large diameter (about 120 μ), pigmentation and apparent thickness (Richards and Walker, '37). Other sites of impalement, the exact location being identified by injection of neoprene, including a few measurements on distal tubule cells, did not give results different from those obtained on upper proximal tubule cells.

In measuring transmembrane potentials of single cells somewhat arbitrary criteria must be applied in estimating the validity of results obtained, particularly when non-excitable tissue is concerned. The following points were taken as criteria for an intracellular location of the electrode tip (Bülbring and Hooton, '54, Kuffler and Vaughan Williams, '53, Nastuk and Hodkin, '50). First, in some preparations and under certain lighting conditions, a mosaiclike structure on the tubule surface suggests cellular borders. From known data on the size of the proximal tubule of Necturus and the width of its lumen (Kempton, '37) one might assume an approximate diameter of 20 μ for cells of the upper proximal tubule. Thus the electrode tip could be advanced directly into this area and visual inspection could be taken as suggestive of cellular puncture. Second, a sudden marked increase in negativity of the microelectrode as it was pushed slowly into the kidney and upon a tubular structure. Third, the potential remained steady rather then fluctuating or declining, indicating effective sealing around the electrode tip. In several instances an increase of the measured potential of a few millivolts occurred immediately following impalement and

is interpreted as progressive sealing around the electrode tip. Potentials were stable as long as 45 minutes, many of them being maintained at the initial level for as long as 10 to 15 minutes. Fourth, ejection of a small amount of colored KCL solution gave no filling of the tubule lumen.

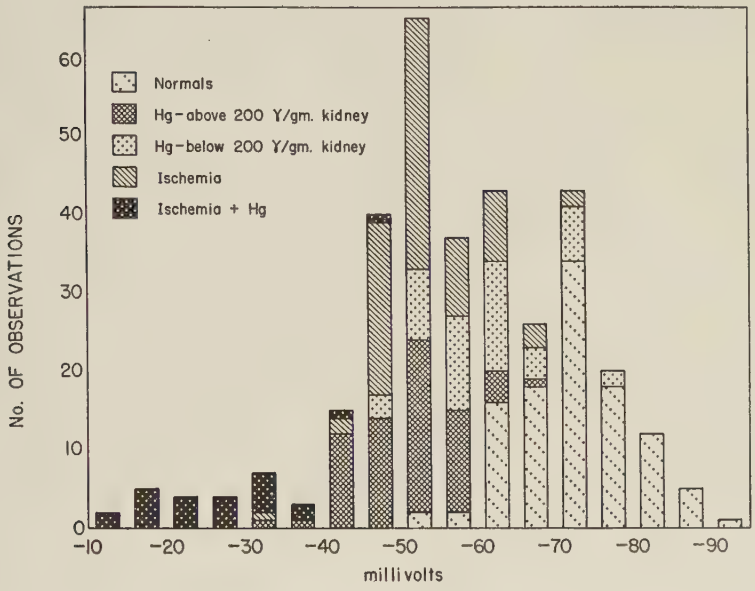


Fig. 1 Summary of transmembrane potentials of single tubule cells. Values of electrical potentials are plotted, pooled in groups of 3 mV, against the number of observations.

In figure 1 are summarized data on the frequency distribution of renal tubular transcellular potentials selected by the criteria mentioned above. Results obtained in animals with undisturbed renal circulation (''normals'') are compared with those having spontaneously a sluggish, poor renal circulation or complete renal vascular stasis (''ischemia''). The results obtained in animals pretreated with various amounts of radio-chlormerodrin (Neohydrin) are also included as well as measurements performed in animals pretreated with the organic mercurial diuretic and having simultaneously poor renal circulation. The results indicate that the mean average

electrical potential difference maintainted across the membrane of single renal tubule cells is – 72 mV (s.d. of the mean ± 7.2 mV). The values ranged from – 54 mV to – 94 mV, the inside of the cell being negative to the outside. The frequency distribution curve peaks smoothly. As can be seen, significantly lower values were obtained in animals pretreated with chlormerodrin in doses which resulted in a mercury content of the kidney exceeding 200 μg/gm kidney. Smaller amounts of mercury (concentration in renal tissue below 200 μg/gm) were less effective in reducing cellular transmembrane potentials. It is furthermore apparent that impairment of the renal circulation also depresses a fraction of the normal membrane potential: as can be seen in figure 1 most potential measurements done on ischemic kidneys yielded values ranging from –45 to –55 mV. We have found that ischemia caused by sluggish blood flow in the superficial capillaries or by complete vascular stasis, and lasting as long as five hours does not lead to a progressive decline of the renal tubular cell potentials. A phase of partial depolarization within the first 30 minutes is followed by stabilization of the potential at a lower level of about 65 to 70% of the original values obtained before ischemia had set in.

Finally the data in figure 1 also show that the lowest potentials were recorded when measurements were done on mercury-pretreated animals having poor renal circulation or complete vascular stasis. Accordingly both variables seem to depress the transmembrane potential in an additive fashion.

The use of radio-chlormerodrin permitted the accurate analysis of the mercurial diuretic in samples of renal tissue from which potential measurements were obtained *in vivo*. In figure 2 are plotted cellular transmembrane potentials as function of renal concentration of radiomercury. Data of untreated control animals are also included in a summarized form. It is apparent that the depression of cellular potentials is greater the larger the concentration of mercury found in the renal tissue.

*Potentials measured across the renal tubule wall of single nephrons.* What we regard as reliable transtubular potential measurements were technically more difficult to obtain than stable cellular potential measurements. The following possibilities of introducing errors should be considered: (1) Indentation of the tubule wall at the site of puncture and

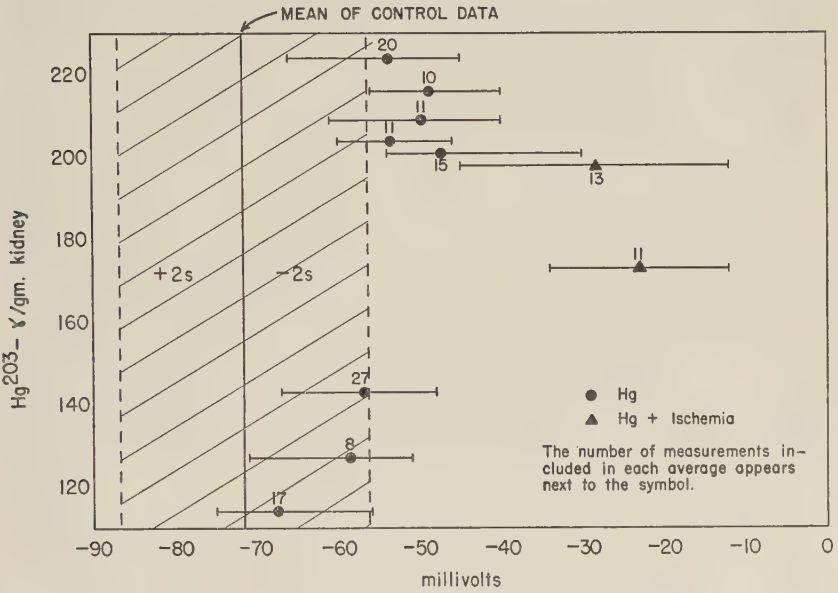


Fig. 2 Renal tubular transmembrane potentials as function of mercury concentration in renal tissue. Each point represents the mean potential difference obtained in mercury-pretreated animals, the range of values being drawn as horizontal line to either side. The straight vertical line at — 72 mV represents the mean average transcellular potential difference in control animals, the broken vertical lines 2 standard deviations to either side of the mean.

mistaking cellular potential differences for transtubular potentials. Ejection of colored KCL solution aids in avoiding this most common error. (2) Since the microelectrode must be advanced across the tubular cell layer and the shaft enlarges immediately behind the tip, local injury (tearing of the tubule wall), shunting and loss of potential difference should be suspected whenever an unstable value is obtained.

In a number of instances we have observed the transtubular potential measurements to be quite sensitive to small changes in tip position. We interpret this as changes in the degree of sealing at the point of impalement where the electrode shaft exceeds the size of the tip. (3) Part of the transtubular potential difference might be shunted by the nephrostome, presumably a low resistance bypass from the neck of the tubule to the kidney surface from which the indifferent electrode leads off. Whether and to what degree this potential loss actually occurs is difficult to assess.

We have obtained 25 potential measurements across single renal tubules which we believe to represent proximal transtubular potential differences and in which to the best of our knowledge the first two sources of errors have been avoided. In all instances the tubule lumen was found to be negative to the outside. The magnitude of the potential difference showed some scatter, the values varying from — 8 mV to — 30 mV (mean : — 20 mV). No differences were observed between upper and lower proximal tubule lumen. In contrast to the findings of Wilbrandt ('38) we have never found the inside of more distal tubule sections to be positive to the outside. Four values obtained from distal tubule lumen (first third) gave values of — 27, — 37, — 39 and — 40 mV. Most of these values are higher than those observed from proximal tubule lumen. In no instance have we been able to obtain an electrical potential difference across the glomerular membrane.

## DISCUSSION

*Transcellular potential differences.* Measurements of electrical potentials across the membrane of single cells of muscle and nerve have shown that the resting transmembrane potential is maintained close to that level which might be expected on the basis of the concentration gradient of potassium maintained across the cell membrane. The latter is believed to be maintained by a metabolically driven sodium extrusion mechanism (Dean, '41). Accordingly the resting potential is principally determined by the difference between

intracellular and extracellular potassium concentration and obtains from the equation $E = \frac{RT}{F} \cdot \ln \frac{K_i}{K_o}$, where $K_i$ and $K_o$ are the concentrations of potassium ions inside and outside. The assumption is made that the activity coefficients of these ion species are the same inside and outside of the cell.

We have measured the concentration ratio $K_i/K_o$ in samples of Necturus kidney to test whether, at physiological concentrations of extracellular potassium, the membrane potential is at or close to the equilibrium potential of potassium. The average value found in our series for intracellular potassium was 132 mEq/L intracellular water (10 animals, range 126 mEq/L — 134 mEq/L), and that of extracellular potassium was 3.22 mEq/L (5 animals, range 2.89 mEq/L to 4.38 mEq/L). Thus an average ratio of 41 obtains for $K_i/K_o$. This ratio can be only a rough approximation because the kidney sample used in the analysis is a collection of cells of different types and does not represent a homogeneous population of proximal tubule cells, such as was used for most of the potential measurements. Uncertainties in the size of extracellular fluid volume do not greatly influence the ratio of $K_i/K_o$. In agreement with others (Manery, '54) we found extracellular potassium to be of the order of magnitude of one per cent or less of total tissue potassium.

Using the Nernst equation the ratio of 41 of $K_i/K_o$ would correspond, at 25°C, to a membrane potential of — 94 mV. While we have observed values as high as this, the majority of our measurements was lower (mean : — 72 mV). The membrane potential of several species of cells has been found to be below the potassium equilibrium potential in the low range of extracellular potassium concentration (Grundfest, '55, Adrian, '56).[2] A number of factors should be considered in accounting for this discrepancy: First, the possibility that

[2] We have explored the possibility of increasing the extracellular potassium concentration by intravenous infusion of potassium chloride solutions, and thus decrease the normal ratio $K_i/K_o$, a reduction which should lead to partial depolarization. However, we were unable, *in vivo* to obtain values above 10 mEq/L without serious cardiovascular side-effects which alone lead to partial break-down of the normal transmembrane potential.

cell injury occurred in our series at the site of impalement cannot be excluded completely. The addition of a small potential depression due to local injury and a small negative junction potential (Adrian, '56) would lead to a reduction of the measured potential difference. Second, the usual assumption that internal and external activity coefficients of potassium are equal is rendered at least questionable in the light of *in vitro* exchange studies of renal tissue which indicate a non-uniform and non-homogeneous intracellular potassium pool (Mudge, '53). Further, there is evidence that *in vitro* the potassium uptake into renal cells is an active process, being independent of sodium extrusion and metabolically conditioned (Mudge, '51, Mudge, '53). Thus if a similar condition obtains for Necturus and if part of the potassium uptake is active this ion would distribute not exclusively according to a Donnan equilibrium. Such a situation would result in an apparent reduction of the cell permeability to potassium and the resulting electrical potential opposing diffusion of potassium out of the cell would be smaller than if all the potassium would distribute solely according to a Donnan equilibrium. Finally, to what extent the distribution of ions other than potassium exerts an influence on the observed potential difference, cannot be assessed acurately at present.

We believe that one or more of the findings just described, while some of them were obtained on species other than Necturus, might account for the deviation observed between actually measured and expected potential difference, were the latter maintained solely on grounds of a Donnan equilibrium for potassium and chloride.

Oxygen lack has been shown to reduce the resting potential of single muscle fibers (Ling and Gerard, '49b, Trautwein, Zink and Kayser, '53) and similar results have been obtained in nerve fibers (Lorente de No, '47). We have also observed a depression of the transcellular potential of single tubule cells after impairment of the renal circulation or after complete renal vascular stasis. It may be of interest that in all

of our experiments only partial depolarization was obtained during periods of ischemia lasting as long as five hours, the membrane potential still being maintained at about 60% of the values obtained before the onset of circulatory deterioration. Most of the reduction of the membrane potential occurred during the first 30 to 50 minutes. Similar results of partial depolarization of the membrane potential of single cells have been obtained in mammalian muscle fibers. Thus, Trautwein and his collaborators ('53) also observed that only part of the membrane potential was depressed after the muscle had been rendered ischemic. In their experiments, as well as in ours, it is difficult to assess what degree of ischemia was accomplished since most punctured cells were superficially located. Diffusion of oxygen through the superficial layer of connective tissue might have accounted for only partial anaerobiosis.

As far as renal tissue is concerned it is noteworthy that the uptake of potassium into renal cells has been found to be dependent on aerobic metabolism (Mudge, '51). The maintenance of a high ratio of $K_i/K_o$ being dependent on an adequate oxygen supply, it would be expected that oxygen lack leads to depression of the membrane potential concomitant with a reduction of the transcellular concentration gradient for potassium ions. Another observation might be relevant. In rabbit kidney slices almost 50% of tissue potassium has been found to be non-exchangeable during oxygen lack when $K^{42}$ was added to the medium. The exchangeability of this moiety of potassium has been demonstrated to be directly dependent on aerobic metabolism (Mudge, '53). Such observations indicate a change in the state of intracellular potassium during oxygen lack which could curtail its participation in a Donnan equilibrium. This also would lead to an apparent reduction of the ratio $K_i/K_o$ and depression of the membrane potential. To what extent ischemic depression of cell metabolism leads to slowing of the sodium extrusion mechanism, is unknown in the kidney of Necturus. However, a gain of sodium by failure of the sodium pump would reduce the internal negative charge

and also lead to loss of polarization of the membrane (Harris, '56).

We have also found that mercury in concentrations of about $10^{-3}$ Mol partially depolarizes single renal tubule cells of Necturus. Blocking of a fraction of the sodium extrusion mechanism, possibly at the basal peritubular end of the tubule cell could explain the partial depolarization of the cell membrane. The fact that mercurial diuretics block only a fraction of the renal tubular reabsorption of sodium is consistent with only partial depolarization of the tubule cell which was actually observed.

An alternative possibility would be a direct influence of the mercurial diuretic on active potassium transport. Indeed, *in vitro* experiments by Mudge ('51, '53) indicate that the uptake of potassium from medium into kidney cells of guinea pigs in independent of sodium extrusion from the cell. Mercurial diuretics, in concentrations of $10^{-3}$ Mol significantly depress the establishment of a high intracellular concentration of this ion and, consequently, would be expected to depress the establishment of a normal transmembrane potential. It is possible that the reduction of oxygen consumption, observed *in vitro* by addition of $10^{-3}$ Mol of a mercurial diuretic (Robinson, '56) contributes also to the observed partial cell depolarization in the manner suggested before.

Since both ischemia and mercurial diuretics per se depress a fraction of the normal transmembrane potential of single tubule cells it is noteworthy that ischemia apparently sensitizes the kidney cells to the effect of mercury: thus the lowest potentials were regularly observed when ischemia was imposed on a kidney poisoned by mercury. Both variables affected the membrane potential in an additive fashion.

*Transtubular potential differences.* Our findings of an electrical potential difference across the proximal tubule wall of single nephrons confirm the earlier results of Wilbrandt ('38) and are in essential agreement with recent observations by Solomon ('57) and Kennedy (personal communication). Obviously an interpretation of the potential difference across

the wall of single tubules is rendered speculative in the absence of simultaneous ion flux measurements across the tubular cell layer (Solomon, Hanenson and Shipp, '56, Hoshiko, Swanson and Visscher, '56). In the following a working hypothesis is presented to explain the maintenance of an electrical potential difference across the proximal tubule wall of single nephrons.

Direct micropuncture studies in Necturus show that as far as sodium, chloride and hydrogen ions are concerned, no concentration gradients are established across the proximal tubule wall between proximal tubule fluid and peritubular extracellular fluid (Bott, '54, Montgomery and Pierce, '37, Giebisch, '56). Therefore an electrical potential difference maintained by an ionic concentration gradient is certainly excluded. Furthermore, there is evidence that sodium chloride is actively reabsorbed from the proximal tubule fluid (Wesson and Anslow, '48), and most authors favor the view, arrived at by indirect evidence, that sodium is the actively transported ion (Smith, '56, Robinson, '54). It seems that in some instances at least, the potassium ion can also be actively reabsorbed from the proximal tubule fluid of Necturus (Bott, '54). This being conceded, a system similar to that proposed by Ussing ('54b) for the maintenance of an electrical potential difference across the frog skin might be envisaged to account for the observed potential difference across the proximal tubule wall of Necturus. If sodium and potassium are actively transported across the tubule, the transport of these ions could generate a potential sufficient for chloride ions to follow passively along the electrical gradient to maintain electroneutrality. The active transport of sodium and potassium would thus create a primary shift of positive charge across the tubule wall, rendering the tubule lumen negative, and producing an electromotive force which attracts chloride ions, and other ions with a negative charge, across the tubule wall. In a steady state the magnitude of the electrical potential will be maintained at such a value which suffices to promote chloride transport at the same speed as that of sodium. The flux of chloride ions would constitute a

shunt for the transtubular potential maintained by the cation pump. Accordingly not only the electromotive force of the sodium and potassium transport mechanism, but also the resistance to the passive flow of chloride ions would determine the magnitude of the transtubular potential difference: chloride permeability and potential difference would then be inversely proportional (Ussing, '54b).

Such a system would create a charge distribution across the single renal tubule similar to that found by us. However is should be stressed that other possibilities such as active transport of positively and negatively charged ions from the tubule lumen to the peritubular fluid at different rates, the transport of water being passive and mediated by the active transport of sodium and chloride, could also account for the observed transtubular potential difference.

## SUMMARY

Electrical potential measurements have been performed on single nephrons of Necturus combining micropuncture techniques with the use of electrolyte-filled glass microelectrodes having a tip diameter of 1 μ or less. Two types of potentials can be distinguished by the use of a microinjection arrangement which permits ejection of a small amount of colored electrolyte solution through the recording microelectrode, thereby allowing for the exact localization of the electrode tip: (1) Transcellular potentials maintained across the cell membrane of single tubule cells and (2) Transtubular potentials maintained between the lumen of single nephrons and the extracellular fluid. The mean transcellular potential difference (mostly early proximal tubule cells) was, in our series, — 72 mV (s.d. ± 7.2 mV), the inside of the cell being negative to the surrounding extracellular fluid pool. These potentials were stable as long as 45 minutes and were significantly depressed by mercurial diuretics and by ischemia. The mean transtubular potential difference obtained across the proximal tubule wall of single nephrons of Necturus was — 20 mV

(range — 8 mV to — 30 mV), the tubule lumen being negative to the outside. The findings are discussed in relation to problems of renal electrolyte transport.

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# RUN-OUT OF CHLORPHENOL RED FOLLOWING LUMINAL ACCUMULATION BY ISOLATED RENAL TUBULES OF THE FLOUNDER *IN VITRO* [1]

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These experiments were undertaken to characterize the process in renal transport which involves the active movement of certain substances against a concentration gradient across the membrane from the proximal cell to tubular lumen (Step 2). Chlorphenol red was used as a representative organic acid capable of undergoing active transcellular movement. Subsequent to maximal intraluminal concentration of the dye, its disposition in the presence of competitive inhibitors and metabolic depressants was observed directly in an *in vitro* system. Earlier studies have disclosed that the cell membrane on the peritubular or vascular side is a site of competitive inhibition among actively transported homologous compounds whose penetration into renal cells (Step 1) is dependent upon certain underlying energy-yielding metabolic reactions (Taggart and Forster, '50; Forster and Taggart, '50; Puck, Wasserman and Fishman, '52; Forster and Copenhaver, '56). Current observations indicate that Step 2 transport across the cell membrane on the luminal side, as penetration on the vascular side, is an energy-demanding step in overall transfer, and an additional site exhibiting competitive inhibition.

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[2] Fellow of the John Simon Guggenheim Memorial Foundation, 1956.

## METHODS

The general procedures used for observing chlorphenol red transfer in this isolated renal tubule preparation were described in an earlier publication (Forster and Hong, '57). The original procedure, developed for the use of phenol red in the study of transport kinetics in thin slices and isolated renal tubules (Forster, '48), was modified to substitute chlorphenol red as the transported compound under observation because of the greater color stability of the latter over the range in pH encountered intracellularly and intraluminally.

Run-out observations were made under uniformly standard conditions. Isolated tubules were oxygenated for 60 minutes at room temperature in a balanced isotonic medium containing $3 \times 10^{-5}$ M chlorphenol red. By this time maximal intraluminal concentration of dye was achieved (++++), and the tubules were then transferred to oxygenated dye-free medium as controls, or to chlorphenol red-free medium modified by the presence of either metabolic depressants or compounds to be tested for their effectiveness as competitive inhibitors. The latter were present in $3 \times 10^{-5}$ M concentration, approximately 1 mg %, simlar to that of chlorphenol red in the original control medium. Some dyes, such as phenol red and bromcresol purple, were found unsatisfactory for this competition study because of difficulty in distinguishing their color from that of chlorphenol red.

## RESULTS

*Metabolic inhibition.* Metabolic inhibitors presented to renal tubules after maximal concentration of chlorphenol red had been achieved facilitated run-out of the dye. General metabolic poisons, such as $HgCl_2$ and NaCN, in concentrations previously shown to depress cellular oxidative processes and inhibit the uptake of organic acid dyes (Forster and Taggart, '50), also interfered with the tubules' ability to retain dye intraluminally. The inhibitory effect was quite prompt, accomplishing 50% run-out within 15 minutes exposure, and

essentially complete leakage back to medium within 30 minutes. Concentrations of 2,4-dinitrophenol, shown earlier to inhibit dye uptake and uncouple aerobic phosphorylation without depressing oxygen uptake by renal tissue (Taggart and Forster, '50), also facilitated run-out. Leakage of chlorphenol red in the cold proceeded faster than at room temperataure, but 60 minutes exposure was required to cause the 50% dye loss from lumen (++) that was noted within 15 minutes with chemical inhibitors. Cold, also, has previously been shown

TABLE 1

*Effect of various metabolic inhibitors on run-out of chlorphenol red from lumen in dye-free medium*

| INHIBITOR | EXPOSURE TIME IN MINUTES | | | |
|---|---|---|---|---|
| | 0 | 15 | 30 | 60 |
| None | ++++ | ++++ | ++++ | +++ |
| Cold (2–4°C) | ++++ | +++ | +++ | ++ |
| 2,4-DNP ($10^{-4}$ M) | ++++ | ++ | ± | — |
| $HgCl_2$ ($10^{-5}$ M) | ++++ | ++ | ± | — |
| NaCN ($10^{-3}$ M) | ++++ | + | ± | — |

Semiquantitative evaluations of dye accumulation in lumen are indicated as ranging from — (no concentration) to ++++ (maximal concentration).

to interfere with dye uptake. These results on dye run-out as affected by metabolic inhibitors are summarized in table 1.

In no instance, whether during dye uptake or during the run-out process, was accumulation noted within cells. Obviously, no intracellular "trapping mechanism" exists for chlorphenol red, such as has been previously shown for certain of the slowly transported members of the phenolsulfonphthalein series (Forster and Hong, '57). The cell membrane on the luminal side is the deterrent to back-diffusion, and once this barrier is removed, leakage of chlorphenol red back to medium is not impeded by the membrane on the peritubular side of the cell nor by any intracellular component momentarily retaining the dye during transit.

The energy requirements of Step I and Step II appear to be identical with respect to dependence upon oxygen uptake and aeorbic phosphorylation. With Step I blocked exclusively, one would still expect the preparation to maintain intraluminal concentrations of chlorphenol red during exposure to metabolic inhibitors in dye-free medium. It seems quite clear that energy is required to maintain the high concentration gradient existing across the luminal cell membrane after active transport of dye has been achieved. Puck, Wasserman and Fishman ('52) have estimated that maximal accumulation in the flounder preparation achieved a 4000-fold lumen: medium concentration when the sustaining fluid contained $2.8 \times 10^{-5}$ M phenol red. The concentration gradient of dye across the luminal membrane is probably greater under current conditions with the tubules in dye-free medium, and no color detectable in the cells. It is reasonable to assume that energy requirements at Step II exceed those calculated earlier for lumen: medium concentration of phenol red where free energy change ($\Delta$ F) per centimeter length of tubule was estimated as $3.0 \times 10^{-6}$ calories.

*Competitive inhibition.* To compare Step I and Step II as sites of competitive inhibition, various substances previously examined for competitive effectiveness at the peritubular site (Forster and Hong, '57) were exposed to tubules subsequent to maximal chlorphenol red accumulation. Originally it seemed reasonable that a second transported substance might occupy some site on a cell membrane "carrier" and through its occupancy would prevent run-out of chlorphenol red from lumen by plugging up the exit route. However, quite the opposite occurred; instead of trapping accumulated chlorphenol red within the lumen, the simultaneous presence of transport competitors facilitated run-out of the dye. As is seen in table 2, *p*-aminohippurate, xylenol blue and bromcresol green were the most effective agents, causing almost complete run-out of accumulated chlorphenol red when the sustaining medium contained $3 \times 10^{-5}$ M concentrations of these competitors. Moderate effects on run-out were noted

with Benemid (probenecid), Diodrast (iodopyrocet), and bromphenol blue. Indigo carmine (a sulfonic acid), bromchlorphenol blue and *p*-aminobenzoate (an analogue of PAH) were ineffective. The effects of cold and competitive inhibitors were not additive in facilitating run-out of chlorphenol red; the simultaneous presence of xylenol blue or bromcresol green with cold did not cause dye to run from the lumen faster than with cold alone.

TABLE 2

*Effect of simultaneous presence of possible competitive inhibitors on run-out of chlorphenol red from lumen in dye-free medium*

| COMPETITOR | EXPOSURE TIME IN CHLORPHENOL RED-FREE MEDIUM | | |
|---|---|---|---|
| | 0 | 30 | 60 |
| | | *min.* | |
| None | ++++ | ++++ | +++ |
| Bromchlorphenol blue | ++++ | ++++ | +++ |
| *p*-Aminobenzoate | ++++ | ++++ | +++ |
| Indigo carmine | ++++ | ++++ | +++ |
| Benemid | ++++ | +++ | ++ |
| Diodrast | ++++ | +++ | ++ |
| Bromphenol blue | ++++ | +++ | ++ |
| *p*-Aminohippurate | ++++ | ++ | + |
| Xylenol blue | ++++ | + | ± |
| Bromcresol green | ++++ | + | ± |
| | Run-out in cold | | |
| None | ++++ | +++ | ++ |
| Xylenol blue | ++++ | +++ | ++ |
| Bromcresol green | ++++ | +++ | ++ |

It is of interest that with members of the phenolsulfonphthalein series effectiveness as competitive inhibitors for uptake was not identical with the order of effectiveness as facilitators of run-out; bromchlorphenol blue, slowly transferred itself, and an effective competitive inhibitor for transport *in vivo* (Sperber, '54) and *in vitro* (Forster, Sperber and Taggart, '54), had no effect on chlorphenol red run-out from the lumen in these experiments. Hence, while both Step I and Step II appear to be sites of competitive inhibition, they seem

to differ from one another with respect to certain factors involved in the competition phenomenon.

If, as seems reasonable, the simultaneous presence of a structural homologue has no effect *per se* on luminal membrane permeability, then the facilitating action of competitors on run-out can perhaps be best interpreted by assuming that a tendency exists for luminally accumulated chlorphenol red to diffuse into the cell, with this passive leakage opposed by an active process which transports dye back into the lumen. Thereby, a steady state is maintained after maximal concentration has occurred, with net luminal accumulation being the resultant of these opposing forces. A "carrier," in or near the luminal cell membrane, capable of association with several transport competitors, might then accept a homologue which displaces chlorphenol red and reduces its active transport into the lumen subsequent to back-diffusion. The fact that the effects of cold and competitive inhibitors are not additive in run-out facilitation could then be explained; the energy-demanding transport process, having been blocked by cold, would not be further affected by competitive inhibition.

## SUMMARY

Metabolic inhibitors and transport competitors were examined in this isolated renal tubule preparation to determine their effectiveness in facilitating run-out of chlorphenol red subsequent to its intraluminal concentration. During uptake or run-out, under control conditions or in the presence of inhibitors, no detectable concentration of chlorphenol red appears in cells.

These studies suggest that the transfer process on the luminal side of proximal tubule cells (Step II) is energy-demanding and subject to competitive inhibition, as is Step I. The latter characteristic, though common to both steps, is not qualitatively identical in Step I and Step II. Maintenance of a high concentration gradient between lumen and cell is viewed

as the resultant of two processes: active transport of dye from cell to lumen, and a slower, passive back-diffusion from lumen to cell.

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# INFRARED SPECTROSCOPY AS A NEW METHOD FOR ASSESSING THE NUTRITIONAL REQUIREMENTS OF THE SLIME MOLD, *DICTYOSTELIUM DISCOIDEUM*

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THREE FIGURES

## INTRODUCTION

During the vegetative stage of its life cycle, the slime mold *Dictyostelium discoideum* feeds upon certain bacteria. Previous work such as that of Raper and Smith ('39) has not developed any relationship differentiating the "edible" from "inedible" bacteria. The usual bacteriological methods have been ineffective for this purpose. Recently, infrared spectroscopy has been applied with varying success to a variety of biological problems. For instance, Stevenson and Bolduan ('52) have shown that whole bacterial cells can be examined rather simply and rapidly and that their infrared spectra are reproducible and sensitive enough to distinguish bacteria of the same species but of different strains. Randall and Smith ('53) also obtained reproducible infrared spectra for biological materials when they applied strict standardization to all procedures. In this research, infrared spectroscopy was used to explore the biochemical relationship between *D. discoideum* and the edible and inedible bacteria.

[1] This author is indebted to the Mycological Society of America for a grant in aid supporting her research.

## MATERIALS AND METHODS

The specimens were prepared for infrared study as thin dried films upon AgCl plates in a manner very similar to that used by Stevenson and Bolduan ('52). The bacteria were grown on the standard medium described by Bonner ('47), on Czapek's agar medium, on potato dextrose agar, or on corn meal agar. Smears were made of the bacterial cultures when they were two days old. Smears were made of *D. discoideum* at the fruiting stage. The spores were spread on the plates to give the necessary thin film. In addition to the usual procedure of joint inoculation, in those instances where *D. discoideum* did not consume the bacteria, the mold was inoculated after the bacteria had grown for two days. This precaution was taken in case the bacteria had not had sufficient time for growth before ingestion by the mold. However, the slime mold did not consume these bacteria in either procedure. Another precaution was taken to ensure that the medium used did not directly inhibit the growth of the slime mold. Those bacteria found to be inedible by the usual procedure were grown separately, then scraped off on 2% agar and inoculated with the amoeba. But here too, the modified procedure did not cause the mold to consume the bacteria.

In order to establish the results as invarient over many generations of the mold and of the bacteria, the infrared spectra of *D. discoideum* and *Escherichia coli* (grown on standard medium) were taken over a period of more than two years. The spectra were recorded with a Perkin-Elmer Model 21 Double beam Spectro-photometer, with a NaCl prism. The spectral region from 4000 to 650 $cm^{-1}$ was observed. A blank AgCl plate was placed in the reference beam to equalize the two light paths.

Quantitative comparisons of absorption band intensities were prevented by the experimental variations in thickness and light scattering of the dried sample films. But, semi-quantitative comparisons were made by using as an internal reference the absorption band appearing at 1650 $cm^{-1}$. Relative absorption intensities, which are theoretically independent of

the sample thickness, were calculated by taking the ratio of the optical density of other absorption bands to that of the reference. These relative absorption intensities for a given band were then compared for different samples.

## RESULTS

The infrared absorption spectra of the whole cells of 12 bacteria are shown in figure 1. The spectra of the first ten bacteria (A-J), *Escherichia coli, Alcaligenes faecalis, Bacillus polymyxa, Brucella bronchiseptica, Micrococcus albus, Micrococcus aureus, Neisseria catarrhalis, Proteus vulgaris, Streptococcus faecalis* and *Serratia marcescens,* are seen to be alike not only in the location of the absorption bands, but also in their relative intensities. These bacteria were found to support growth of the slime mold (see table 1). On the other hand, the spectra of *Bacillus megatherium* and *Sarcina lutea* (K and L) include major absorption bands not found for the other bacteria. These bacteria did not support growth of *D. discoideum.* The main differences in the spectra are indicated by arrows.

Figure 2 presents a typical infrared spectrum of *D. discoideum* as grown upon *E. coli.* This spectrum of *D. discoideum* is indistinguishable from the spectra of samples grown upon the other edible bacteria.[2] The spectra of *E. coli* and of the medium are also given in figure 2 for comparison. It is seen that the spectrum of the *D. discoideum* is nearly identical with the spectra of the first ten bacteria, as shown by the comparison with *E. coli.*

In figure 3 are presented spectra of four different bacteria, each of which was grown upon two different media. Even though spectra A and B are both of *A. aerogenes,* they are quite different. *A. aerogenes* grown upon standard medium was able to support growth of the mold whereas *A. aerogenes* grown upon Czepek's agar medium was not able to support

[2] *D. discoideum* has the same infrared spectrum as the bacteria long after the cessation of feeding. The mold had completed the processes of feeding, migration, differentiation and fruiting when the spectra were taken.

growth. Similarly *B. subtilis* grown on potato dextrose agar, *M. citreus* grown on standard medium, and *Ps. aeruginosa* grown on standard medium were all able to support growth of the mold; whereas, *B. subtilis* on standard medium, *M. citreus*

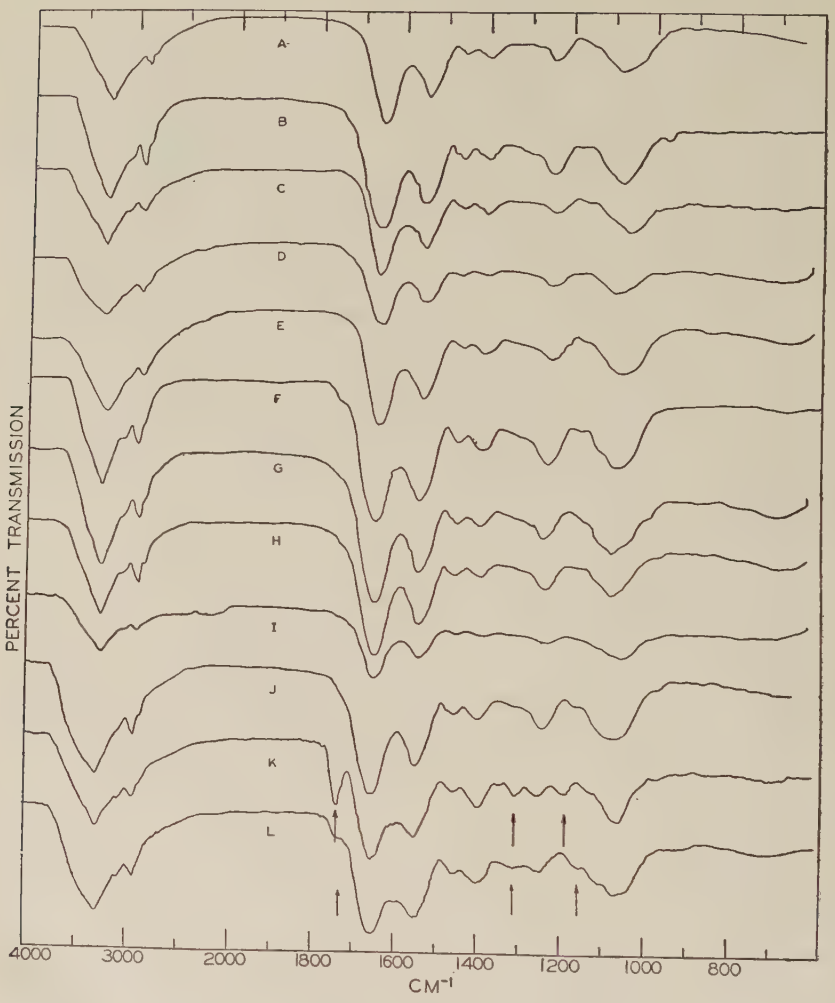


Fig. 1 Infrared absorption spectra of different bacterial species cultured for 48 hours on Bonner's standard medium. (A) *E. coli.* (B) *Alc. faecalis.* (C) *B. polymyxa.* (D) *Br. bronchiseptica.* (E) *M. albus.* (F) *M. aureus.* (G) *N. catarrhalis.* (H) *P. vulgaris.* (I) *Str. faecalis.* (J) *S. marcescens.* (K) *B. megatherium.* (L) *S. lutea.*

TABLE 1

*Summary of bacteria, similarity of their infrared spectra to that of* D. discoideum, *and the growth of the latter upon the bacterial cultures*

| CULTURE | MEDIUM | SPECTRUM OF CULTURE AND OF D. DISCOIDEUM | GROWTH OF D. DISCOIDEUM UPON CULTURE |
|---|---|---|---|
| *A. aerogenes* | Standard [1] | Similar | Good |
| *A. aerogenes* | Czapek's [2] | Different | None |
| *Alc. faecalis* | Standard | Similar | Good |
| *B. polymyxa* | Standard | Similar | Good |
| *B. subtilis* | Potato dextrose [2] | Similar | Good |
| *B. subtilis* | Standard | Different | None |
| *B. megatherium* | Standard | Different | None |
| *Br. bronchiseptica* | Standard | Similar | Good |
| *E. coli* | Standard | Similar | Good |
| *M. citreus* | Standard | Similar | Good |
| *M. citreus* | Corn meal [2] | Different | None |
| *M. albus* | Standard | Similar | Good |
| *M. aureus* | Standard | Similar | Good |
| *N. catarrhalis* | Standard | Similar | Good |
| *P. vulgaris* | Standard | Similar | Good |
| *Ps. aeruginosa* | Standard | Similar | Good |
| *Ps. aeruginosa* | Potato dextrose [2] | Different | None |
| *S. lutea* | Standard | Different | None |
| *Str. faecalis* | Standard | Similar | Good |
| *S. marcescens* | Standard | Similar | Good |

[1] Standard — Bonner's standard medium. [2] These are agar media.

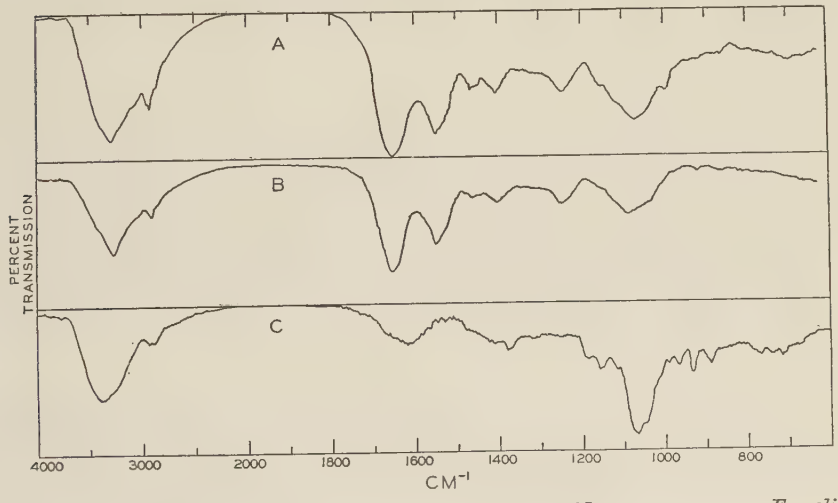


Fig. 2 Infrared absorption spectra of (A) *D. discoideum* grown upon *E. coli*, (B) *E. coli* and (C) Bonner's standard medium.

on corn meal agar and *Ps. aeruginosa* grown on potato dextrose agar were not able to support growth of *D. discoideum*. In all cases where the bacterium was nutritionally suitable for the mold, its infrared spectrum was similar to that of the mold.

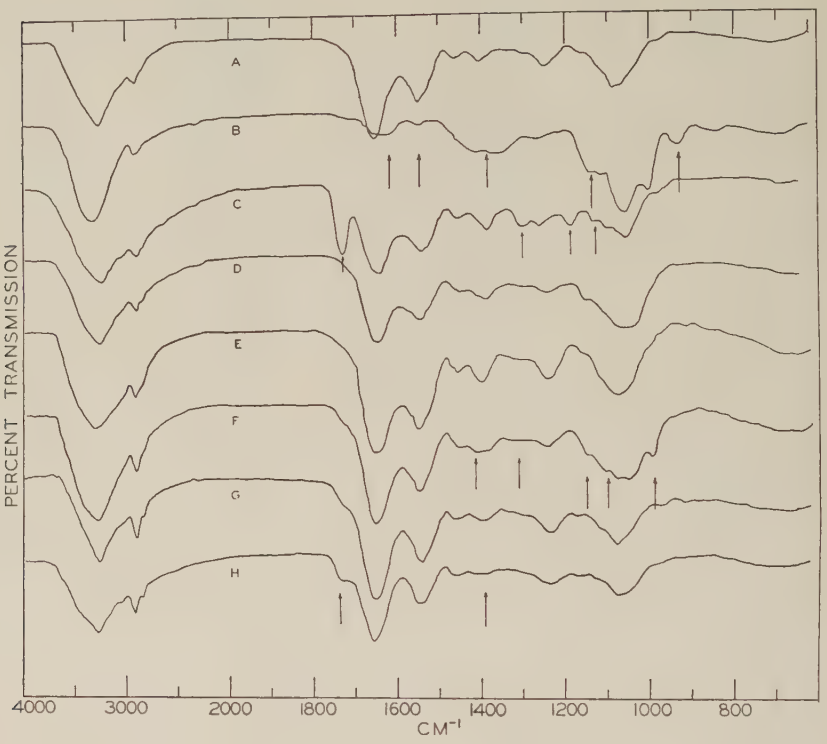


Fig. 3 Infrared absorption spectra of (A) *A. aerogenes* grown on standard medium, (B) *A. aerogenes* grown on Czapek's agar medium, (C) *B. subtilis* grown on standard medium, (D) *B. subtilis* grown on potato dextrose agar, (E) *M. citreus* grown on standard medium, (F) *M. citreus* grown on corn meal agar, (G) *Ps. aeruginosa* grown on standard medium, and (H) *Ps. aeruginosa* grown on potato dextrose agar.

Particular chemical groups normally give infrared absorption at characteristic frequencies. The main absorption bands of *D. discoideum* are characteristic of poly-peptides, nucleic acids and carbohydrates, as shown in table 2 by the assign-

ments of the bands to particular structural groups, following in main the assignments of Levine et al. ('53) for *E. coli.*

The inedible bacteria, *B. megatherium* and *S. lutea,* have absorption bands very similar in postion and relative intensity to all of the other bands of the edible group. In addition *B. megatherium* has a sharp strong band at 1740 $cm^{-1}$ and weak bands at 1320 and 1190 $cm^{-1}$ while *S. lutea* has a rather weak shoulder at 1730 $cm^{-1}$ and weak bands at 1310 and 1155 $cm^{-1}$. These bands are indicated by arrows in figure 1.

TABLE 2

*Structural assignments for the characteristic infrared absorption bands of* D. discoideum *and the edible bacteria*

| ABSORPTION BAND | DESCRIPTION | ASSIGNMENT |
|---|---|---|
| 3300 $cm^{-1}$ | strong, broad | N-H and O-H stretching |
| 3060 | weak, shoulder | N-H stretching (poly-peptide) |
| 2920 | weak | C-H stretching |
| 2840 | very weak shoulder | C-H stretching |
| 1650 | very strong | C = O stretching (peptide) |
| 1545 | strong | N-H bending (peptide) |
| 1450 | weak | C-H bending (methylene) |
| 1400 | medium weak | C-H bending (?) |
| 1240 | medium | O-H bending (?) (nucleic acids) |
| 1050 | strong, broad | C-O and N-O stretching (?) (nucleic acids and carbohydrates) |

The absorption at 1740 and 1730 $cm^{-1}$ in the inedible bacteria reveals the presence of C=O groups other than the amide groups which absorb at 1650 $cm^{-1}$. Infrared spectra of compounds of known structure, such as given by Barnes et al. ('44), characterize the 1700 to 1750 $cm^{-1}$ region as typical of ester carbonyl groups. Many esters also show two weaker absorption bands, one near 1300 $cm^{-1}$, the other near 1200 $cm^{-1}$ and the bands observed at 1320 and 1190 $cm^{-1}$ in *B. megatherium* support the occurrence of ester groups as a significant factor in this species. The biochemistry of *S. lutea* must be somewhat different since the three extra bands in it are at consistently lower frequencies than the corresponding bands in *B. megatherium.*

In the case of *Ps. aeruginosa* on potato dextrose agar, the extra (ester) bands are relatively much weaker; the band at about 1300 $cm^{-1}$ is not visable at all. Therefore, the ester groups are not as major a component as in the case of the other inedible bacteria, but the difference is still great enough to make the *Ps. aeruginosa* unsuitable. The spectrum of *A. aerogenes* on Czapek's agar medium reveals a deficiency as much as a difference in composition. The peptide bands at 1650 and 1545 $cm^{-1}$ are almost completely missing. The N-H and O-H bands are at a somewhat higher frequency than 3300 $cm^{-1}$. The strong absorption between 1150 and 1000 $cm^{-1}$ could arise from any of a number of things (perhaps carbohydrates).

The extra bands between 1310 and 995 $cm^{-1}$ shown by *M. citreus* grown on corn meal agar are all present to a lesser degree for the edible bacteria. In *M. citreus* they are sharper and more intense, suggesting that the relative amounts of its chemical components are different than in the edible bacteria. That is, *M. citreus* has more nucleic acids and carbohydrates.

## DISCUSSION AND CONCLUSIONS

The positions and relative intensities of the absorption bands in an infrared spectrum are sensitive indications of the gross chemical composition of the sample. It was found that only those bacteria with a specific infrared spectrum were nutritionally suitable for the amoebae of *D. discoideum.* When this specific infrared spectrum was altered by changing the medium on which the bacteria were inoculated, the mold would no longer consume the bacteria. Likewise, when a dissimilar spectrum (as in *B. subtilis*) was changed to this characteristic spectrum, the bacteria would be eaten. In addition, the spectra of the edible bacteria could not be distinguished from the spectrum of *D. discoideum,* demonstrating that the main chemical components of the mold are very similar to those of the edible bacteria.

It is of interest to speculate on the significance of the similarity in the spectra of the slime mold and of the bacteria it consumes. It is possible that the edibility of the bacteria

is determined by some unknown factor or mechanism which is associated in a non-causal manner with the similarity of the infrared spectra. On this basis, the similarity found in the infrared spectra is of only incidental importance. There is also the simpler and more intriguing possibility that the experimental results reflect a true causal relationship between the biochemical constitution of the edible bacteria and *D. discoideum*. This latter hypothesis is attractive and seems well worth further testing. In particular, studies might be made of problems such as host specificities and the nutritional requirements of parasites.

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# *IN VITRO* TRANSPORT OF DYES BY ISOLATED RENAL TUBULES OF THE FLOUNDER AS DISCLOSED BY DIRECT VISUALIZATION. INTRACELLULAR ACCUMULATION AND TRANSCELLULAR MOVEMENT [1]

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These experiments were undertaken to describe by direct observation the disposition of certain representative acidic and basic organic dyes during their transport across renal tubule cells, especially with respect to competition and energy dependence. The over-all transcellular process was considered as involving, first, movement of dye into the cell from the peritubular fluid (Step I), and second, transfer out of the cell with subsequent further concentration within the lumen (Step II). For some dyes, an intermediate "trapping mechanism" appeared to constitute a separate phase. In contrast to thin slices of mammalian renal cortex, which *in vitro* exhibit only Step I as an active process, the isolated fish tubule preparation used here, in addition transports certain organic acid dyes across the luminal cell membrane against concentration gradients (Forster, '48; Forster and Taggart, '50; Forster, Sperber and Taggart, '54; Forster and Copenhaver, '56).

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## METHODS

The procedures used were generally similar to those described in earlier publications (Forster, '48; Forster and Taggart, '50), except that chlorphenol red was used instead of phenol red in control experiments. The bluish-red color of the former is more readily perceived, and its color stability within the range of pH encountered in these preparations is also a distinct advantage (Forster, Sperber and Taggart, '54). Of the various fishes tested for these *in vitro* studies the readily available flounder was found best; its kidney, composed exclusively of proximal (brush border) tubules and almost completely devoid of lymphoid tissue, presents a uniform appearance when teased and viewed under the microscope, and is most viable under *in vitro* conditions.

Several modifications of the basic technique introduced by others (Puck, Wasserman and Fishman, '52; Wasserman, Becker and Fishman, '53) to improve the quantitative determination of phenol red uptake were found of no value when applied to chlorphenol red studies, especially elevated temperature and high bicarbonate concentration in the sustaining medium. Comparators may be used to ascribe absolute values to dye concentrations within cells or lumina, but questionable assumptions need then be made concerning such factors which may interfere with light absorption as depth of standard, depth of structure in kidney containing dye, and the presence of extraneous cellular factors which modify color. In our experience, the most satisfactory procedure for the semiquantitative evaluation of dye concentration in cells and lumina has been the assignment of arbitrary concentration ratings ranging from + (definitely detectable) to ++++ (maximal), in accordance with the behavior of the majority of tubules. Surprising uniformity in interpretation can be achieved by several observers after some practice, and actually eight useful ratings can be made between zero and maximum concentration.

Freshly captured flounder were broken open after an incision was made through the vertebral column in the area of

the gills, and within several seconds small fragments of renal tissue were transferred to an oxygenated, balanced, isotonic salt solution which had been empirically derived for its ability to sustain secretory activity in renal tubules (Forster, '48). This solution contained the following concentrations of salts in millimoles per liter: NaCl 135, KCl 2.5, $CaCl_2$ 1.5, $MgCl_2$ 1.0, $NaH_2PO_4$ 0.5, and $NaHCO_3$ 10. It is best to add sodium bicarbonate as dry salt while stirring, after water has been added to the mixture. A phosphate-buffered solution without bicarbonate ion does not sustain maximal transport of dye. If 5% $CO_2$ — 95% $O_2$ is used rather than pure oxygen the mixture stabilizes within the pH range 7.0 — 7.4, whereas with 100% $O_2$ pH climbs in time as high as 8.5. The dye transport process, however, is not affected by the variations in pH obtained with either oxygenation procedure.

Petri dishes (1.2 × 4 cm) containing 5 ml of the sustaining medium were used to observe dye transport in teased kidney fragments under 100 × magnification. Usually 7 such Petri dishes, containing $3 \times 10^{-5}$ M concentration of the various dyes in the medium described above, were oxygenated simultaneously via 22 gauge hypodermic needles at laboratory temperatures. Oxygenation was interrupted momentarily when dishes were transferred to the microscope stage from time to time for evaluation of dye concentration in cells or lumina.

Two special methods based on earlier studies were examined as procedures which might be used in the characterization of Step II. Wasserman, Becker and Fishman ('53) reported a "self-blockade" phenomenon that resulted when phenol red concentration in the medium was raised to 40 mg % and above. As a consequence high concentrations of dye were achieved intracellularly and none was detectable in the lumen. Their use of phenol red necessitated a high bicarbonate concentration in the medium (40 mM) and a relatively high temperature (28° C) in order to provide quantitative evaluation of the phenol red color. Under the conditions of the current experiment the "self-blockade" phenomenon could not be demon-

strated with either flounder or Fundulus tubules. When limitations of transfer were obtained in the presence of very high phenol red and chlorphenol red levels (120 mg %), inhibition was imposed at Step I, which resulted in significantly less total uptake of dye. Only rarely was any detectable intracellular accumulation noted ($< ++$), and then the luminal dye concentration was significantly greater than that present simultaneously in the cells. This type of inhibition is viewed as being relatively non-specific, and of no value in assessing characteristics of membrane phenomena with respect to Step II transport.

However, dependence of the Step II transport process on calcium ions (Puck, Wasserman and Fishman, '52; Wasserman, Becker and Fishman, '53) has been confirmed in the current studies as a relatively specific phenomenon. In calcium-free medium, phenolsulfonphthaleins accumulated intracellularly with no subsequent transfer to the lumen, and when, in addition, potassium was eliminated from the medium, movement of dye into the cells was blocked. The dependence of Step I on potassium was further disclosed by the proportional increase of intracellular dye uptake when potassium concentrations in calcium-free media were increased progressively from zero to 15 mM. Inhibition was reversed when kidney fragments were transferred from a calcium-free medium to the balanced medium to the extent that the intracellular dye moved to the lumen in about one-half the tubules within 50 — 60 minutes. A critical time factor which has not been thoroughly examined may be involved in this reversal of Step II inhibition. Further evidence for the relative specificity of Step II dependence on calcium ions was gained from respiration studies which disclosed that oxygen uptake *in vitro* by the flounder kidney was not affected by absence of calcium in the sustaining medium, even when potassium concentrations were raised as high as 15 mM. Under these circumstances, with Step II activity blocked, and Step I activity potentiated, very high intracellular concentrations of dye were achieved with no subsequent transfer into the lumen.

## RESULTS

*Similarity of phenol red and chlorphenol red transport characteristics.* Earlier studies *in vivo* and *in vitro* have disclosed that chlorphenol red is excreted by vertebrate renal tubules in generally the same manner as is phenol red (Sperber, '54; Forster, Sperber and Taggart, '54). Additional detailed observations were made here on isolated renal tubules, which indicate further that these phenolsulfonphthaleins are handled identically in each of the separate steps involved in the transfer process, and that chlorphenol red should be used instead of phenol red for direct observations of this kind because of the stability of its color over the range in pH encountered both intracellularly and intraluminally in these preparations. These dyes competed for transport reciprocally; they accumulated within the lumen at identical rates; after accumulation they ran out similarly when treated with cold or 2,4-dinitrophenol; transfer rates for both were independent of pH in the range examined (6.5 – 9.0); neither accumulated intracellularly during transport except in the absence of calcium; and both required bicarbonate ion for maximal excretion. The balanced isotonic medium containing phosphate buffer (pH 7.5) without bicarbonate sustained only moderate uptake (++). Maximal transfer rates (++++) were attained with 10 mM sodium bicarbonate in the medium with the use of either 100% oxygen or a 5% $CO_2$ – 95% $O_2$ mixture (pH 7.3). The transfer rates of neither dye were affected by the presence of equimolar concentrations of a simultaneously transported basic ion, e.g. tetraethylammonium.

*The effect of general metabolic inhibitors on uptake of various organic dyes.* The effect of cold (2 – 4°C) was determined as follows. The dishes containing freshly teased fragments of renal tissue were placed in an ice bath; the isolated tubules were examined for dye concentration in cells and lumina after 20, 60 and 120 minutes in the cold. The results are shown in table 1. Cold and dinitrophenol (Forster and Taggart, '50; Taggart and Forster, '50), acting as general

TABLE 1

*Accumulation of various dyes in cold*

| TIME (Min.) | CONTROL (20°C) | | IN COLD (2 - 4°C) | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Chlor-phenol red | | Phenol red | | Chlor-phenol red | | Xylenol blue | | Brom-chlor-phenol blue | | Brom-cresol purple | | Brom-cresol green | | Brom-phenol blue | | Neutral red | | Indigo carmine | |
| | *L* | *C* | *L* | *C* | *L* | *C* | *L* | *C* | *L* | *C* | *L* | *C* | *L* | *C* | *L* | *C* | *L* | *C* | *L* | *C* |
| 20 | +++ | — | — | — | — | — | — | — | — | — | — | — | ± | + | + | ++ | ++ | — | — | ± |
| 60 | ++++ | — | ± | — | ± | — | ± | — | ± | — | ± | — | ++ | ++ | +++ | +++ | ++++ | — | — | ± |
| 120 | ++++ | — | ± | — | ± | — | ± | — | ± | — | ± | — | ++ | ++ | +++ | +++ | ++++ | — | ± | — |

The oxygenated isolated tubule preparations were incubated in a cold bath with the balanced isotonic salt solution in each instance containing $3 \times 10^{-5}$ M concentrations of the dyes. L = concentration of dye in lumen, and C = concentration within cells.

depressants, had identical inhibitory effects on uptake of the various dyes whose transport characteristics were examined. Among the phenolsulfonphthaleins, those transported most actively normally (phenol red, chlorphenol red, xylenol blue, bromchlorphenol blue and bromcresol purple) were inhibited most effectively by cold and DNP, while the sluggishly transported members of the series, (bromcresol green and bromphenol blue) were affected relatively little, or not at all, by these metabolic inhibitors. The slow-moving members of the series also showed the greater tendency to accumulate intracellularly. Transfer of the basic dye, neutral red (Kempton, '39), which was rapidly concentrated in the lumen, was not affected by these metabolic inhibitors. On the other hand, transport of the sulfonic acid dye, indigo carmine (Ponceau R), (Höber and Briscoe-Woolley, '40), was completely blocked, whereas normally it too was transported very rapidly. The special feature of indigo carmine uptake was that dye concentrated first in cells and only later in the lumina. At the end of 30 minutes dye in cells had reached +++ while intraluminal concentrations were ++; tubules transferred then to dye-free medium under control conditions slowly passed intracellularly accumulated indigo carmine along into the lumen. This Step II process was inhibited by cold (2 – 4°C). Acetate, which has been shown to stimulate active transport of *p*-aminohippurate and certain phenolsulfonphthaleins (Cross and Taggart, '50; Forster and Copenhaver, '56), was ineffective in facilitating transfer of sulfonic acid dye from cell to lumen.

These observations suggest that transported compounds may be grouped into several categories based on their transfer characteristics with respect to movement via passive penetration or active transport. Furthermore, selective differences exist among them concerning their relative dependence on Steps I or II as either energy-demanding actions or passive process effecting transfer.

*Run-out subsequent to concentration.* In the following experiments isolated tubules were exposed to the various dyes

($3 \times 10^{-5}$ M) under control conditions for 60 minutes, and then transferred to oxygenated dye-free medium for another 60 minutes. Run-out observations were then made on tubules kept under refrigeration (2 – 4°C), and on control preparations maintained at laboratory temperature (20°C). The dyes were grouped into three categories with respect to their run-out characteristics. First, those phenolsulfonphthaleins which were transported most actively, and whose uptake was most effectively blocked by metabolic inhibitors (phenol red, chlorphenol red, xylenol blue and bromcresol purple), showed the greatest tendency to run out when transferred to dye-free medium in the cold. Typically, at the end of the 60 minute run-out period luminal concentrations dropped from ++++ to ++ in the cold and to +++ at room temperature with no discernible accumulation within cells. In the second category were the slowly transported phenolsulfonphthaleins (bromcresol green and bromphenol blue) and the basic dye neutral red. These showed no loss of color in the dye-free medium after 60 minutes, whether maintained at room temperature or in the cold. Run-out behavior of the sulfonic acid, indigo carmine, represented the third situation where, subsequent to accumulation (luminal and intracellular), dye was lost at room temperature but not in the cold. These observations are summarized in table 2.

*Intracellular "trapping."* From the above observations on uptake and run-out, inferences can be drawn concerning certain energetic relations and the possible existence of intracellular carriers capable of holding transported substances with varying degrees of firmness. The intracellular component in the transfer process may act as a selective solvent or adsorbant, or as an intermediate entering into chemical reaction with the transported compound; certainly these alternatives are not mutually exclusive. Whatever the "trapping mechanism" may be, it is clear that within a series of homologous compounds capable of being transferred across tubule cells those members which are not transported actively show the greatest tendency to accumulate intracellularly, and they

TABLE 2

*Summary relating the characteristics of various dyes (phenolsulfonphthaleins, basic dyes and sulfonic acid derivative) to uptake by isolated renal tubules, the effectiveness of general metabolic inhibitors on uptake, and run-out subsequent to 60 minute accumulation at room temperature and in the cold*

| DYE | MOL. WT. | pK | NORMAL UPTAKE AFTER 60 MIN. | | UPTAKE IN THE PRESENCE OF COLD OR DNP | | RUN-OUT IN DYE-FREE MEDIUM AFTER ACCUMULATION | |
|---|---|---|---|---|---|---|---|---|
| | | | *Lumen* | *Cell* | *Lumen* | *Cell* | *20°C* | *2-4°C* |
| Phenol red | 354 | 7.8 | ++++ | − | ± | − | slow | fast |
| Chlorphenol red | 424 | 6.2 | ++++ | − | ± | − | slow | fast |
| Xylenol blue | 382 | 8.0 | ++++ | − | ± | − | slow | fast |
| Bromcresol purple | 540 | 6.3 | ++++ | − | ± | − | slow | fast |
| Bromphenol blue | 670 | 4.0 | +++ | +++ | +++ | +++ | none | none |
| Bromcresol green | 698 | 4.7 | ++ | ++ | ++ | ++ | none | none |
| Neutral red (basic dye) | 289 | ca. 7.4 | ++++ | − | ++++ | − | none | none |
| Cyanine #863 (basic dye) | 390 | | ++++ | +++ | ++++ | +++ | * | * |
| Indigo carmine (sulfonic acid) | 466 | ca. 12.5 | +++ | ++++ | − | ± | medium | none |

* Toxicity damages cells during 60 minute accumulation period and precludes run-out observations.

are the most effective inhibitors of others in the series more actively transported (see also Sperber, '54; Forster, Sperber and Taggart, '54). Hence, phenol red, chlorphenol red, xylenol blue and bromcresol purple were transported very actively and the concentration gradient between cell and lumen was maintained by a high level of metabolic activity; Step II proceeded at a faster rate than Step I so no intracellular accumulation occurred during transcellular movement. When metabolism was interfered with, as by transfer of tissue to dye-free medium in the cold, these dyes ran back to the ambient medium without being trapped intracellularly.

On the other hand, bromcresol green and bromphenol blue, which previously were shown to block very effectively the more actively transported members of the series, accumulated intracellularly by passive diffusion even in the presence of metabolic inhibitors; once trapped within the cells they did not run out when the tubules were transferred to dye-free medium at room temperature or in the cold. The resulting intracellular color was distributed uniformly and no selective accumulation in vacuoles or on particulate matter was discernible at the 100 $\times$ magnification used in these studies. In general, it might also be noted that as far as phenolsulfonphthalein dyes are concerned, those with low molecular weight and high pK tended to be transported actively, while those with high molecular weight and with low pK were transported passively and tended to be trapped within the cell (table 2).

The basic dye, neutral red, appeared also to be transported by some energy-independent process, but in contrast to bromcresol green and bromphenol blue it was concentrated within the lumen and appeared to stain some structural component at the brush border, imparting a spotty effect rather than the even distribution of color noted with the phenolsulfonphthaleins. Again, it showed no tendency to run out after the tubules were transferred to dye-free medium in the cold.

The disulfonic acid, indigo carmine (Ponceau R), occupies an intermediate position in the sense that while Step I and Step II both appeared to be energy-demanding, movement

into the lumen proceeded relatively slowly, and dye was trapped within cells where some of it stained the nucleus and certain cytoplasmic inclusions. After uptake for 60 minutes when cellular concentration approximated that in the lumen, transfer of the tissue to dye-free medium in the cold resulted in "trapping" all the indigo carmine within the cells with no discernible movement into the lumen or run-out back into the ambient medium. Incidentally, indigo carmine uptake was not affected by simultaneous presence of chlorphenol red in equimolar concentration whereas *p*-aminohippurate, and especially Probenecid, competitively inhibited transport of the dye.

*The basic dye cyanine no. 863.* This dye has recently received considerable attention as a representative of a series of bases which are excreted *in vivo* and *in vitro* by a mechanism different from that concerned in the transport of *p*-aminohippurate, the phenolsulfonphthaleins, and the organic acids (Peters, et al., '55; Rennick, et al., '56). The flounder tubule preparation took up cyanine very fast; the dye accumulated intracellularly where it behaved as a vital stain, in some ways similar to neutral red. Cold and 2,4-dinitrophenol did not inhibit uptake, nor did they facilitate run-out after accumulation had occurred. It did not appear to concentrate luminally, but visualization was difficult because of the very strong intracellular accumulation. Cyanine was quite toxic to renal tubule cells in the $3 \times 10^{-5}$ M concentration used for these experiments, which precluded run-out observations after 60 minute exposure periods, as were made with other dyes (table 2). This dye, which has been shown to block effectively the excretion of other bases such as tetraethylammonium and N'-methylnicotinamide, was transported slowly and "trapped" intracellularly. This reciprocal relationship between competitive inhibition and intracellular accumulation resembles that noted in the phenolsulfonphthalein series with bromcresol green and bromphenol blue.

## GENERAL DISCUSSION

The objective of this series of experiments was merely to describe the behavior *in vitro* of various acidic and basic dyes with respect to their disposition in a renal tubular system capable of actively transporting certain of these substances transcellularly. No attempt will be made at this time to delineate physicochemical characteristics of the compounds which might account for their selective treatment by the secretory system. Interpretation of cellular transport processes, when they effect transfer against electrochemical gradients, is complicated by the polyphasic nature of the system.

Three separate phases seem to be involved, each one of which may or may not be energy-demanding. First, entry into the cell may occur by passive penetration (e.g. neutral red, bromphenol blue and cyanine dye), but substances transported across renal cells at a rapid rate (e.g. *p*-aminohippurate, phenol red and chlorphenol red) appear to enter via an energy-demanding step at the peritubular cell membrane. This is also the site of competitive inhibition, as has been demonstrated in the flounder tubule preparation with calcium-free medium and in rabbit kidney cortex slices maintained *in vitro* (Forster and Copenhaver, '56). For actively transported compounds movement into the cell is the rate-limiting step in over-all transfer across renal tubules *in vitro*. Secondly, the tendency for a compound to accumulate and become "trapped" intracellularly is inversely related to the rate at which the substance is excreted, and directly related to its effectiveness as a competitive inhibitor. Intracellular accumulation may result possibly from selective adsorption or solvency, or it may be due to slow turnover rates in some stoichiometrical reaction involved in transport. Finally, a step in the transfer process takes place at the membrane on the luminal side of the cell which accomplishes further concentration of certain dyes within the lumen. This step may proceed in the presence of general metabolic inhibitors, as with neutral red, or it may be dependent upon a relatively high rate of metabolic activity, as with the actively trans-

ported organic acids. With the latter this transfer across the luminal membrane in the isolated fish tubule preparation proceeds at a faster rate than that which moves these substances into the cell, so no intracellular accumulation is detectable during transport across cells. It is sensitive to metabolic inhibitors, and when the step is blocked by cold and DNP subsequent to accumulation, these dyes quickly run out of the lumen back into the ambient medium without accumulating intracellularly. Active transfer of organic acid dyes across the luminal side of the cell membrane does not take place *in vitro* with rabbit cortex slices (Forster and Copenhaver, '56). It has not been resolved whether this discrepancy in dye disposition is due to species differences, or to undisclosed manipulative procedures used in handling mammalian tissue slices, which selectively affect Step II activity without interfering with active intracellular uptake.

## SUMMARY

Certain representative acidic and basic dyes have been observed directly while undergoing cellular transport in an isolated renal tubule preparation *in vitro*. For compounds capable of being actively transported across renal cells, movement into cells from ambient medium is the rate-limiting process; subsequent concentration in the lumen then proceeds without detectable intracellular accumulation. However, in an homologous series such as the phenolsulfonphthaleins, those compounds not transported actively tend to accumulate within tubule cells, and they are more effective as competitive inhibitors than are the more actively transported substances in such a series. The behavior of various dyes during uptake and run-out under experimentally imposed conditions supports the view that transport across renal cells involves at least three phases, each of which, for the different dyes, may or may not be dependent upon energy-yielding metabolic events. These include the two steps which move substances across the peritubular and the luminal membranes, and an intracellular

"trapping mechanism" exhibited most prominently with those basic and acidic compounds which are least actively transported.

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# PERMEABILITY AND UTILIZATION OF GLUCOSE IN MAMMALIAN ERYTHROCYTES [1]

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ELEVEN FIGURES

Mammalian erythrocytes other than those of man and ape were considered to be impermeable to glucose by the earliest workers to investigate the problem. Numerous studies of partition of "blood sugar" between cells and plasma demonstrated that concentrations were much higher in the plasma than in the cells (Andreen-Svedberg, '33). When glucose was added to whole blood, it could be recovered almost entirely from the plasma (Masing, '12; Kozawa, '14; Ege, '20). Neither volume changes nor hemolysis could be detected when the cells were exposed to isosmotic glucose solutions (Kozawa, '14; Ege, '21; Ulrich, '34). However, Wilbrandt ('38) with a method which depended upon a change in osmotic resistance with the entrance of hexose demonstrated a low degree of permeability to hexoses in rabbit, rat and dog cells. More recently Morgan et al. ('55) have reported that the movement of glucose into rabbit erythrocytes cannot be explained by simple diffusion and have suggested an enzyme-catalyzed or carrier system. A high degree of permeability for glucose has been demonstrated in the foetal cells of the so-called impermeable species (Widdas, '55).

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The glycolytic capacity of the impermeable cells and differences among species in this capacity are well known (Loeb, '13; Engelhardt and Ljubimowa, '30). Two hypotheses have been proposed to account for the metabolism of apparently impermeable cells. Andreen-Svedberg ('33) suggested the possibility that simple diffusion just balanced metabolism so that the cells remained glucose free. Others favored the concept developed by Hober (Kozawa, '14) of a permeability dependent on cellular activity (physiological permeability).

No one has carefully studied glucose utilization and permeability in the same system over some time period. The effects of glycolytic inhibitors on the utilization and permeability in different species are unknown. It is believed that such studies would be of value in elucidating the problem of the glucose permeability of the apparently impermeable mammalian erythrocytes.

## METHODS

The method of collection of blood varied with the species employed. Rabbit and pig samples were obtained by heart puncture; sheep, dog and human by veni-puncture; and rat, guinea pig and beef by exsanguination. The blood of several animals had to be pooled in experiments with rats and guinea pigs in order to have a sufficient quantity. The work reported here involving pig, dog and sheep blood was done using blood obtained at intervals from a single animal of each species. The results with human blood described were obtained from a single sample of blood. After collection the blood was defibrinated and filtered through gauze. Sterile precautions were observed during all experiments lasting longer than five hours, except those with rat and guinea pig blood.

An attempt was made to remove cells other than erythrocytes in all experiments with rat and guinea pig bloods and in two instances (designated "white cells removed" in the text) with rabbit blood. In these experiments the blood was centrifuged at 3000 G for approximately twenty-five minutes and the plasma was withdrawn. The buffy coat and top

layers of red cells were then removed and the plasma remixed with the erythrocytes. Whole blood was used in the other experiments reported below.

If glucose or other substances were to be added to a volume of blood, the compounds were dissolved in a portion of the plasma of that blood sample, and this portion was then readded to the portion containing the cells.

The blood samples were incubated at 37°C in glass containers whose capacity was at least five times that of the sample volume. The large containers afforded the air space necessary to keep changes in the gaseous environment of the samples to a minimum. Adequate mixing was insured during incubation by intermittent rolling, fifteen minutes in motion followed by fifteen minutes at rest (Green and Parpart, '53). At various time intervals, 0.2 $cm^3$ of plasma, obtained by centrifugation of 1 $cm^3$ of blood at 17,000 G for fifteen minutes with an air turbine centrifuge (Parpart and Green, '51) and 0.2 $cm^3$ of whole blood were diluted with 5 $cm^3$ of distilled water. The diluted samples were treated with the protein precipitants described by Nelson ('44) and then stored overnight at 5°C for subsequent glucose analysis. Hematocrits were determined at the same time using the technique of Parpart and Ballentine ('43).

Experiments were performed to measure the hexokinase activity of whole hemolysates and stroma free hemolysates. Cells were packed for thirty minutes at 0°C with a Servall centrifuge at 16,000 RPM. After the plasma and white cells were discarded, the erythrocytes were frozen and thawed twice. To every volume of red cells 3 volumes of a solution containing 0.013 M $Na_2HPO_4$ — $NaH_2PO_4$ (pH 7.4), .06 M $MgCl_2$, .01 M NaF and approximately 100 mg % glucose were added. A portion of the diluted hemolysate was then centrifuged in the Servall at 16,000 RPM for thirty minutes to yield stroma free hemolysate. ATP was added to some samples just prior to incubation at 37°C.

Analyses of glucose content were done according to the method described by Nelson ('44) as modified for use of the Beckman spectrophotometer by Hempling ('54).

pH determinations of the whole blood were made with a Cambridge glass electrode pH meter.

## RESULTS

The permeability of erythrocytes for glucose was measured in these experiments as the loss of glucose from the plasma, i.e., the maximum amount of glucose which could have entered the cells. The glucose values obtained by chemical analyses yield values of the concentrations of glucose in the plasma and the whole blood. These data have been treated in the following way in order to calculate the maximum amount of glucose that might have entered the red cells in a standard system of whole blood. Let the volume of the whole blood be 100 $cm^3$. The amount of glucose in the whole blood will be distributed in some manner between the plasma and cellular compartments of the blood. The number of milligrams of glucose present in each compartment can be calculated:

$$\text{plasma conc. of glucose expressed as mg/100 } cm^3 \text{ plasma} \times \text{the fraction of whole blood that is plasma} = \text{mg glucose located in the plasma of the 100 } cm^3 \text{ whole blood system}$$

$$\text{mg glucose in 100 } cm^3 \text{ whole blood} - \text{mg glucose located in the plasma of the 100 } cm^3 \text{ whole blood system} = \text{mg glucose in the red cells of the 100 } cm^3 \text{ whole blood system}$$

After losses from the plasma and the whole blood were determined, a further correction of these values was made to standardize the initial hematocrit to 40%:

$$\frac{0.40}{\text{initial red cell fraction}} \times \text{loss from whole blood or plasma at some time} = \text{corrected value}$$

Corrections to a 40% hematocrit have been made on all of the losses from the whole blood and plasma presented below unless noted otherwise.

One can also express these data as concentrations of glucose in plasma or red cell water. In these calculations the initial values for the percentage water of the plasma and red cells

are assumed to be 92 and 65 respectively. Determinations of the percent water content of the erythrocytes used in these investigations vary (Ponder, '48) and 65% was selected as an average value.

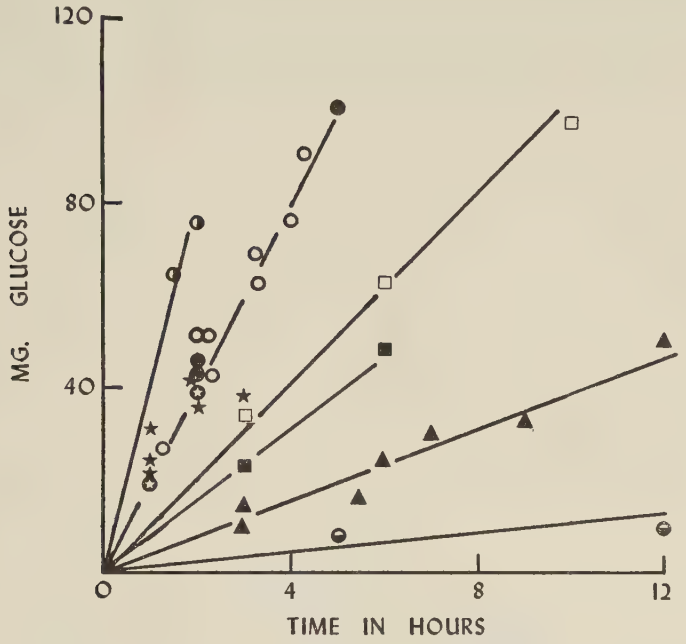


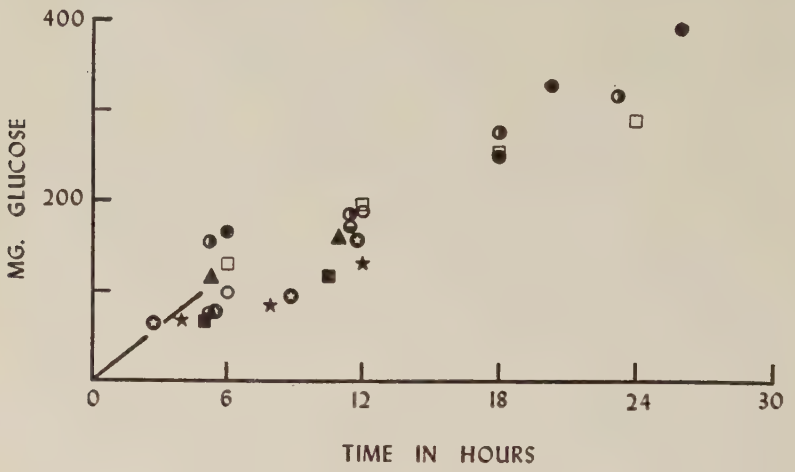

Fig. 1 The loss of glucose from 100 cm³ of whole blood (40% hematocrit initially) of various species when no glucose has been added to the blood. ◐ = Rat; ○ = Rabbit (white cells present); ● = Rabbit (white cells removed); ★ = Dog; ✪ = Human; □ = Guinea Pig; ■ = Sheep; ▲ = Beef; ◒ = Pig.

## RABBIT

*Glucose utilization.* The glucose utilization by the red cells of five samples of rabbit blood is presented in figure 1. The buffy coats and top layers of red cells were removed in two samples to determine whether the white cells participated in and/or influenced the utilizations of glucose by whole blood. No difference was evident between whole blood and blood from which a large proportion of white cells was removed. The rate of utilization, 53 mg/100 cm³ cells/hr., remained constant

over a four hour experimental period despite the continuous fall in glucose concentration.

The utilization of glucose upon addition of various amounts of glucose to seven samples (A, B, C, D, E, F, G) of rabbit blood is described by figure 2. It can be seen that the rate of utilization during the first six hours was higher than that of the remainder of the experiment. There is considerable variation among experiments at the 500 mg % level of glucose con-




Figs. 2 and 3 Rabbit: The loss of glucose from 100 cm³ of whole blood (upper graph) and from the plasma of this blood (lower graph) when various amounts of glucose have been added to the blood. In the following, the letter indicates the sample of blood and the number the initial concentration of glucose in mg %. □ = A, 527; ◒ = B, 508; ◑ = B, 930; ○ = C, 598; ● = C, 2600; ▲ = D, 534; ✪ = E, 563; ★ = F, 588; ■ = G, 540. The line describes glucose loss from whole blood when no glucose was added.

centration, but the utilization during the first four hours was similar to that of the samples to which no glucose had been added.

*Glucose distribution and factors affecting it.* Determinations of glucose concentrations in plasma and erythrocyte water showed the initial concentrations to be higher in the plasma (vide table 1). Additions of glucose were made to aliquots of a blood sample (C) to raise the initial whole blood glucose concentrations to 598 mg % (500 mg % added) in one aliquot

and to 2600 mg % (2500 mg % added) in another. As seen in table 1, the glucose content of the red cells of these aliquots increased during incubation, but the plasma concentrations exceeded those of the cells throughout the experiments.

*The loss of glucose from the plasma.* The losses of glucose from the plasma following the additions of glucose are shown in figure 3. These losses increase as the amount of glucose supplied is raised. The losses observed upon addition of

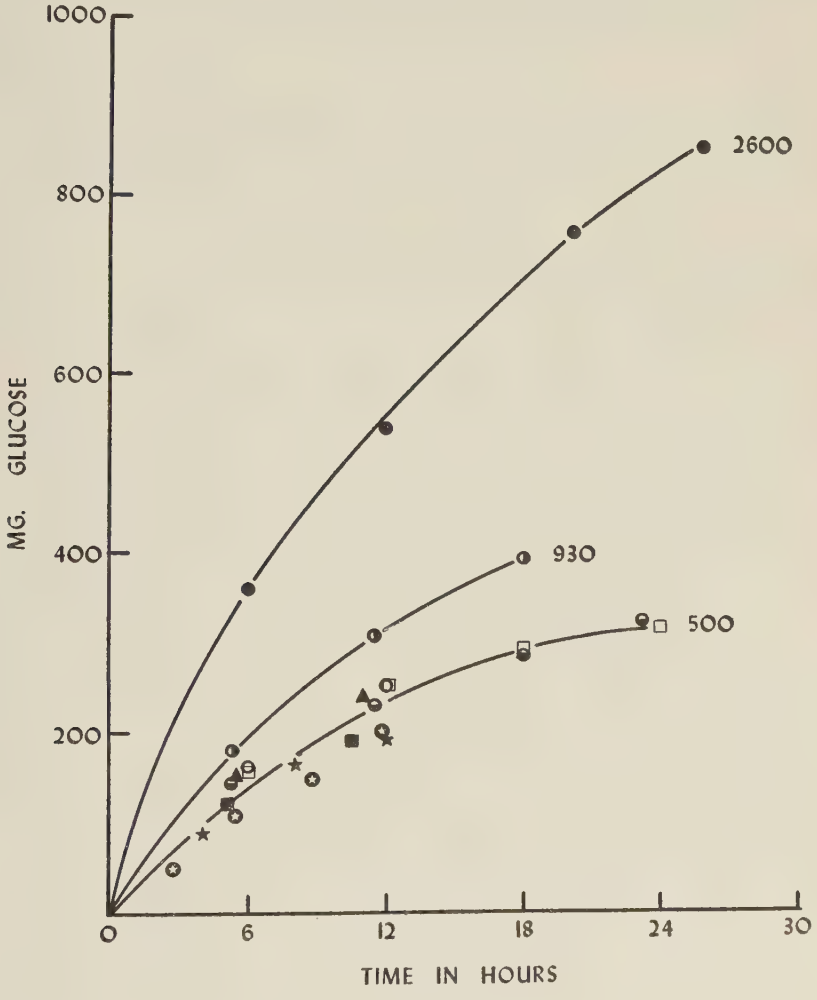



Figure 3

500 mg % to six different blood samples demonstrate the variability among individuals of this species.

*Effect of metabolic inhibitors.* Metabolic poisons were employed in an attempt to simplify the study of glucose permeability by removing the complication of utilization. Cellular concentrations after incubation with 500 mg % and 2200 mg %

TABLE 1

*Rabbit: The concentration of glucose in cells and plasma expressed as mg of glucose/100 cm³ of water in cells and plasma*

| SAMPLE | LENGTH OF INCUBATION HOURS | MG OF GLUCOSE/100 CM³ OF WATER OF THE | |
|---|---|---|---|
| | | PLASMA | CELLS |
| | No glucose added to the blood | | |
| A | 0 | 162 | 35 |
| B | 0 | 130 | 56 |
| C | 0 | 163 | 55 |
| | 500 mg glucose added to the blood initially | | |
| C | 0 | 1120 | 59 |
| | 6 | 798 | 309 |
| | 12 | 584 | 306 |
| | 2500 mg glucose added to the blood initially | | |
| C | 0 | 4350 | 100 |
| | 6 | 3890 | 1050 |
| | 20 | 3350 | 2190 |
| | 26 | 3120 | 2000 |
| | 500 mg glucose and .001 M IAA added to the blood initially | | |
| D | 0 | 998 | 16 |
| | 5¼ | 752 | 392 |
| | 11 | 712 | 480 |
| | 2200 mg glucose and .001 M IAA added to the blood initially | | |
| H | 0 | 3510 | 174 |
| | 2 | 3390 | 680 |
| | 4 | 3270 | 1120 |
| | 8¼ | 3170 | 1540 |
| | 12 | 3120 | 1770 |
| | 22 | 2920 | 2340 |
| | 500 mg glucose and .005 M NaF added to the blood initially | | |
| F | 0 | 966 | 71 |
| | 4 | 860 | 385 |
| | 8 | 767 | 550 |
| | 12 | 746 | 440 |

glucose and .001 M Iodoacetic Acid (IAA) are seen in table 1. Although these concentrations approached those of the plasma, they were always lower even after incubations of twenty-two hours. Yet in comparison with no IAA present, the amount in the cells is higher at any given time.

The demonstration of shrinkage of erythrocytes in the presence of IAA by Wilbrandt ('40) was confirmed. This shrinkage could not be simulated by the addition of 0.001 M acetic acid or 0.001 M trichloroacetic acid to the blood, and would not be expected from earlier observations (Jacobs and Parpart, '31).

Glucose movements similar to those obtained with 0.001 M IAA were observed when glucose metabolism was inhibited with 0.005 M NaF. The change in cellular glucose concentration after addition of 500 mg % glucose and 0.005 M NaF is also shown in table 1.

The influence of a third compound, phloridzin, was studied at a concentration of this compound at which *no* inhibition of utilization occurred. At this concentration, 0.004 M, the loss of glucose from the plasma and the volume changes of the phloridzin treated cells were smaller than those of the control samples. The pH changes were similar in the two samples, and therefore, pH was eliminated as a possible factor in the anomolous volume changes. The whole blood and plasma glucose losses, the volumes and the pH values shown in figure 4 were obtained from aliquots of the blood sample E. At a higher concentration, .01 M, phloridzin would inhibit glucose metabolism (fig. 4).

A further study was conducted to determine whether the differences in volume changes of the control and phloridzin-treated samples were due entirely to the lower cellular glucose concentrations—and hence lower osmotic pressures in the latter samples—or to some other factor. Volume and pH changes of the whole blood with and without 0.004 M phloridzin were investigated. In these experiments differences in cellular glucose concentrations were eliminated because of the low concentrations of glucose in the systems. The data

presented in table 2 show that phloridzin must influence the volume of the erythrocytes, as the volume of the control and treated cells differed after five hours of incubation. The differences in volume of the red cells between the samples,

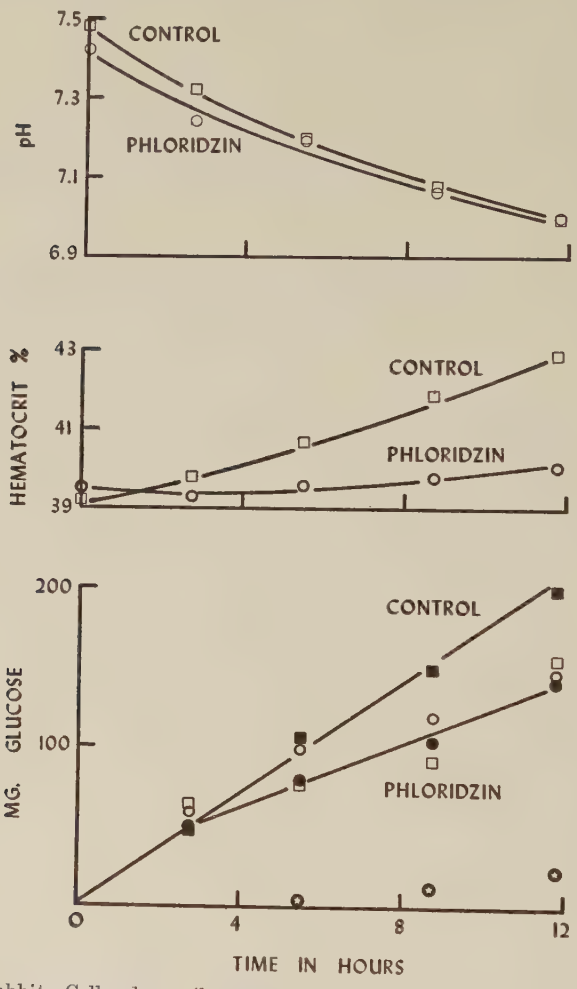



Fig. 4 Rabbit: Cell volume (hematocrit), pH and the loss of glucose from the whole blood and plasma of 100 cm³ whole blood (40% hematocrit initially) after addition of 500 mg % glucose in the presence (circles) and absences (squares) of phloridzin. In lower curve the blackened symbols are loss from plasma, while open symbols are loss from whole blood. Glucose loss from whole blood in the presence of .01 M phloridzin is shown by open star in dark circle.

though small, are significant because of the high reproduceability of the hematocrit method employed (Parpart and Ballentine, '43).

Rabbit blood was incubated with 0.01 M sucrose and 500 mg % glucose to test the possibility that the phloridzin effect might be the result of the presence of a large "inert" molecule. However, glucose losses from the plasma and whole blood were identical in the samples containing sucrose and the controls.

TABLE 2

*Rabbit: The effect of .004 M phloridzin on the volume of red cells*

| TIME | CONTROL | | PHLORIDZIN | |
|---|---|---|---|---|
| | Hematocrit | pH | Hematocrit | pH |
| *Hours* | | | | |
| 0 | 40.0 | 7.63 | 40.5 | 7.60 |
| 1 | 40.2 | 7.58 | 40.3 | 7.57 |
| 3 | 40.6 | 7.53 | 40.5 | 7.51 |
| 5 | 41.3 | 7.42 | 40.7 | 7.42 |
| 7 | 41.4 | 7.38 | 40.8 | 7.39 |
| 8½ | 41.6 | 7.36 | 40.4 | 7.41 |

RAT

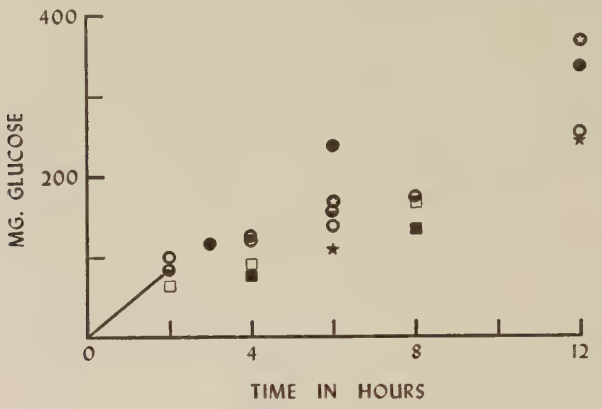
*Glucose utilization.* The average initial rate of glucose utilization of rat blood when no addition of glucose was made to the samples was 101 mg/100 $cm^3$ cells/hr. (fig. 1).

The variations in the glucose utilization over a range of glucose concentrations are shown in figure 5. Four samples of rat blood were used (A, B, C, D). The initial utilization rate was not changed by the addition of glucose to the samples. This rate was not maintained, however, and generally decreased less rapidly in the samples at concentrations of ca. 2500 mg % glucose than in others.

*Glucose distribution and factors affecting it.* The initial glucose distribution of one sample (B) of fresh rat blood is shown in table 3. When glucose was added to a sample of blood, cellular concentrations increased during incubation. The distributions of glucose in aliquots of a sample of blood

(B) to which glucose was added are recorded in table 3. It can be seen that cellular glucose concentrations approach those of the plasma at twelve hours.

*The loss of glucose from the plasma.* The dependence of glucose loss from the plasma on blood glucose levels can be seen in figure 6. Losses of glucose from the plasma increase when the plasma glucose concentrations are raised.




Figs. 5 and 6 Rat: The loss of glucose from 100 cm³ whole blood (upper graph) and from the plasma of this blood (lower graph) when various amounts of glucose have been added to the blood. In the following, the letter indicates the sample of blood and the number the initial concentration of glucose in mg %. ◒ = A, 204; ○ = A, 644; ● = A, 2785; ★ = B, 598; ✪ = B, 2680; □ = C, 565; ■ = D, 549. The line describes glucose loss from whole blood when no glucose was added.

*Effect of metabolic inhibitors.* Although the concentrations of NaF and IAA which would stop glucose utilization in rabbit cells had no effect on rat cells, inhibition was attained with an increase in the NaF concentration to 0.03 M. When the blood glucose concentration was raised to 801 mg % (700 mg % added) in the presence of 0.03 M NaF, the cellular glucose concentration rose and equalled that of the plasma in four hours, as seen in table 3. Increases in the concentration of IAA to 0.003 M led to hemolysis and the action of this compound was not investigated further.

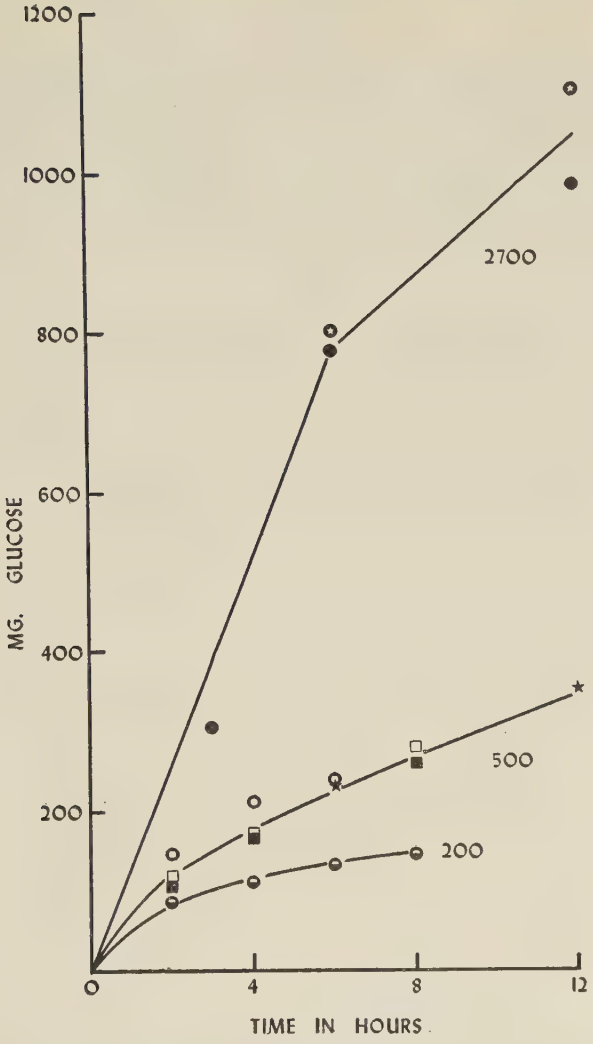



Figure 6

The effects of 0.004 M phloridzin observed with rabbit blood were also found in rat blood. The results of two experiments with phloridzin are shown in figure 7. The plasma glucose losses and volume changes of the phloridzin-treated cells were smaller than those of the control, while the whole blood glucose losses and pH changes showed no differences.

DOG

*Glucose utilization.* The glucose losses from whole blood to which no glucose was added are shown in figure 1. The average rate of three blood samples was 54 mg/100 $cm^3$ cells/hr. In order to study the influence of concentration on glucose utilization, glucose was added to aliquots of two of these samples of

TABLE 3

*Rat: The concentration of glucose in the cells and plasma expressed as mg of glucose/100 $cm^3$ of water in the cells and plasma*

| SAMPLE | LENGTH OF INCUBATION HOURS | MG OF GLUCOSE/100 $CM^3$ OF WATER OF THE | |
|---|---|---|---|
| | | PLASMA | CELLS |
| | No glucose added to the blood | | |
| B | 0 | 114 | 78 |
| | 500 mg glucose added to the blood initially | | |
| B | 0 | 1050 | 25 |
| | 6 | 667 | 445 |
| | 12 | 452 | 394 |
| | 2500 mg glucose added to the blood initially | | |
| B | 0 | 4290 | 0 |
| | 6 | 3360 | 2440 |
| | 12 | 3030 | 2560 |
| | 700 mg glucose and .03 M NaF added to the blood | | |
| E | 0 | 1285 | 105 |
| | 1 | 1170 | 500 |
| | 2 | 1060 | 800 |
| | 3 | 1000 | 965 |
| | 4 | 995 | 990 |

blood. The results of these experiments seen in figure 8 show that glucose utilization did not vary in the three concentrations investigated. The initial rate obtained from the "glucose added" samples appears to be lower than that of the "no glucose added" samples (fig. 1). Since the first determinations were made at five hours with the "glucose added" cells and at one hour with "no glucose added" cells, it is possible that this difference is merely a reflection of the general decrease

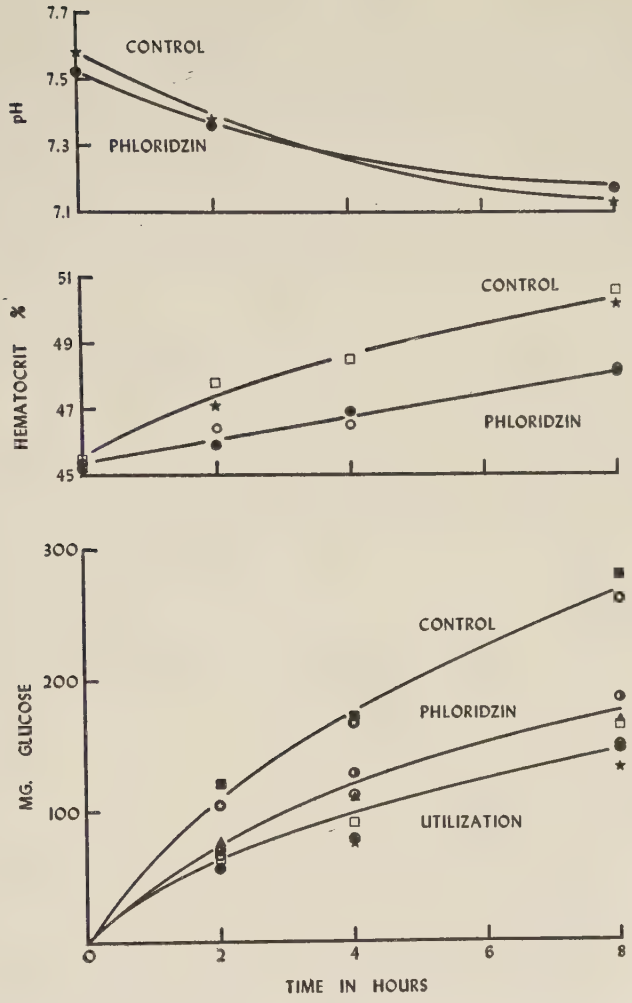



Fig. 7 Rat: The pH, cellular volume (hematocrit) and the loss of glucose from the plasma and whole blood (utilization) of 100 cm³ whole blood (40% hematocrit initially) after addition of 500 mg % glucose in the presence and absence of .004 M phloridzin.

| | CONTROL | | .004 M PHLORIDZIN | |
|---|---|---|---|---|
| Blood sample | C | D | C | D |
| mg glucose loss from whole blood, hematocrit and pH | □ | ★ | ○ | ● |
| mg glucose loss from plasma | ■ | ✪ | ◑ | ▲ |

in utilization rates with time observed in these and other cells studied.

*Glucose distribution.* The plasma and cellular concentrations of glucose after addition of glucose to the whole blood

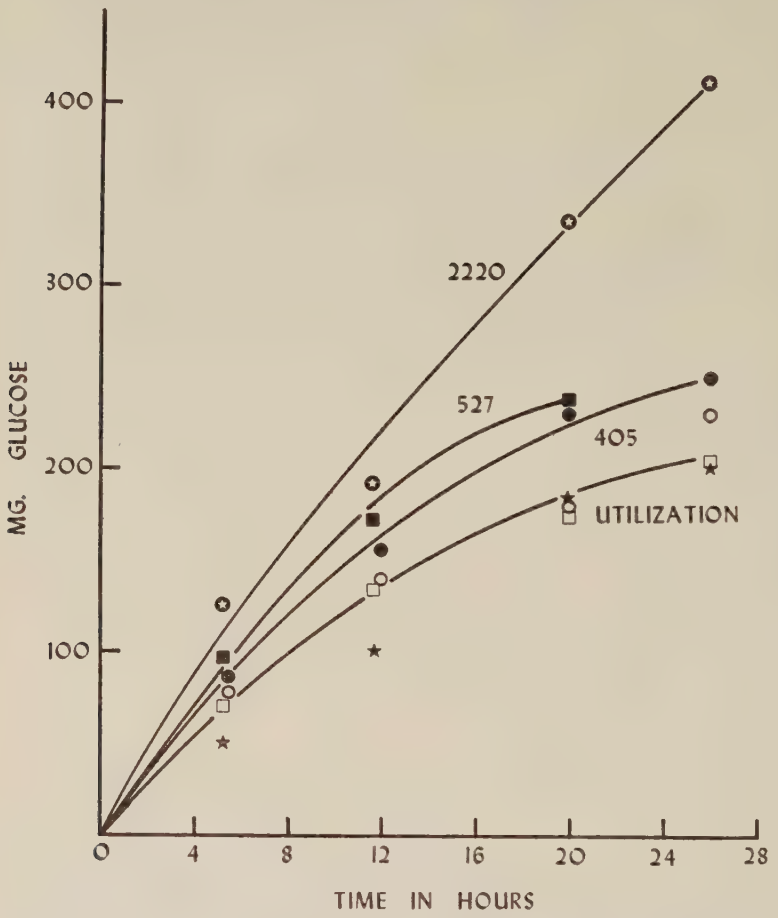



Fig. 8 Dog: The loss of glucose from the plasma and whole blood (utilization) of 100 cm³ of whole blood after addition of various amounts of glucose to the blood.

| BLOOD SAMPLE | INITIAL GLUCOSE CONCENTRATION MG/100 CM³ BLOOD | MG GLUCOSE LOSS FROM | |
|---|---|---|---|
| | | Whole blood | Plasma |
| A | 405 | ○ | ● |
| B | 527 | □ | ■ |
| B | 2220 | ★ | ✪ |

are shown in table 4. Although there is a continuous increase in the cellular concentration, plasma concentrations exceed those of the cells throughout the experimental period.

*Glucose loss from the plasma.* The losses of glucose from the plasma in the aliquots of samples A and B to which glucose was added are also shown in figure 8. Although the glucose utilization in these aliquots did not vary, the plasma losses increased as the amounts supplied were raised.

TABLE 4

*Dog: The concentration of glucose in the cells and plasma expressed as mg of glucose/100 cm³ of water in cells and plasma*

| SAMPLE | LENGTH OF INCUBATION HOURS | MG OF GLUCOSE/100 CM³ OF WATER OF THE | |
|---|---|---|---|
| | | PLASMA | CELLS |
| | 500 mg glucose added to the blood initially | | |
| B | 0 | 987 | 82 |
| | 5¼ | 793 | 183 |
| | 11¾ | 633 | 219 |
| | 20 | 479 | 304 |
| | 2500 mg glucose added to the blood initially | | |
| B | 0 | 3680 | 106 |
| | 5¼ | 3320 | 636 |
| | 11¾ | 3210 | 743 |
| | 20 | 2960 | 1090 |
| | 25¾ | 2850 | 1430 |

*Effects of inhibitors.* An attempt was made to study glucose movements in the presence of 0.001 M IAA and 0.005 M NaF. These inhibitors produced marked hemolysis within 5 hours, however, and this line of investigation was abandoned (see also Wilbrandt, '40).

GUINEA PIG

*Glucose utilization.* The glucose utilization of guinea pig erythrocytes was determined in two samples (A and B) of blood. From the whole blood glucose losses shown in figure 9, it can be seen that the rate of utilization of glucose was inde-

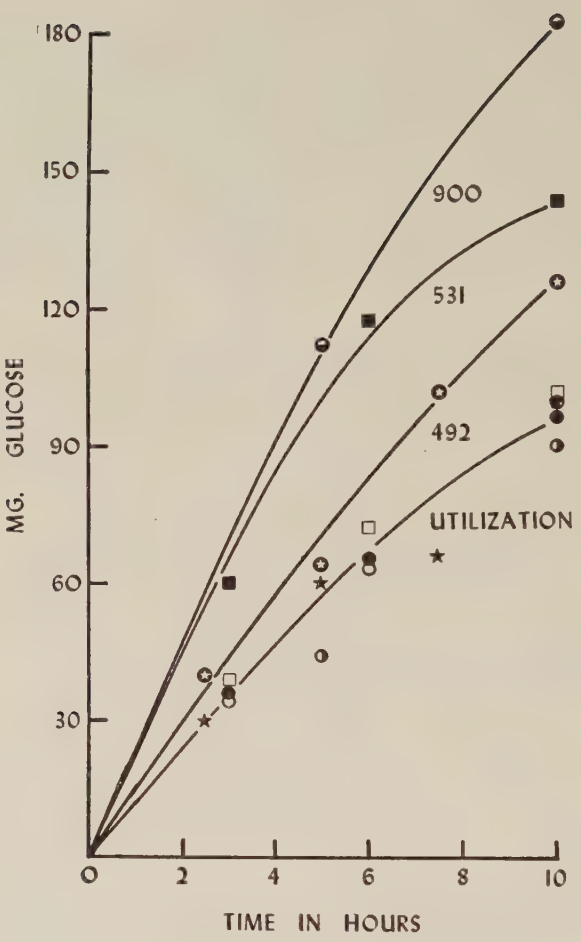



Fig. 9 Guinea pig: The loss of glucose from the plasma and whole blood (utilization) of 100 cm³ of whole blood after addition of various amounts of glucose to the blood.

| BLOOD SAMPLE | INITIAL GLUCOSE CONCENTRATION MG/100 CM³ BLOOD | MG GLUCOSE LOSS FROM | |
|---|---|---|---|
| | | Whole blood | Plasma |
| A | 135 | ○ | ● |
| A | 531 | □ | ■ |
| A | 900 | ◑ | ◒ |
| B | 492 | ★ | ✪ |

pendent of the glucose concentration in the range investigated. The glucose utilization rate was 26 mg/100 $cm^3$ cells/hr.

*Glucose distribution.* The plasma and cellular concentrations of glucose of the three aliquots of one sample of blood are shown in table 5. In the aliquots to which glucose was added, the cellular glucose concentrations increased with time but remained lower than those of the plasma throughout the ten hour experimental period.

TABLE 5

*Guinea pig: The concentration of glucose in cells and plasma expressed as mg of glucose/100 $cm^3$ of water in cells and plasma*

| SAMPLE | LENGTH OF INCUBATION HOURS | MG OF GLUCOSE/100 $CM^3$ OF WATER OF THE | |
|---|---|---|---|
| | | PLASMA | CELLS |
| | No glucose added to the blood | | |
| A | 0 | 244 | 43.7 |
| | 500 mg glucose added to the blood initially | | |
| A | 0 | 995 | 44 |
| | 3 | 880 | 130 |
| | 6 | 764 | 218 |
| | 10 | 721 | 188 |
| | 800 mg glucose added to the blood initially | | |
| A | 0 | 1680 | 0 |
| | 5 | 1490 | 281 |
| | 10 | 1380 | 332 |
| | 400 mg glucose and .03 M NaF added to the blood initially | | |
| B | 0 | 829 | 0 |
| | 2½ | 765 | 120 |
| | 5 | 767 | 161 |
| | 7½ | 737 | 277 |
| | 10 | 737 | 268 |

*Glucose loss from the plasma.* The plasma glucose losses from the samples (same as those described above) to which glucose was added are shown in figure 9. An increase in plasma glucose losses occurred upon addition of glucose to the blood.

*Effect of inhibitors.* The increases in cellular glucose concentrations while glucose utilization was inhibited with 0.03 M

NaF are shown in table 5 (400 mg % glucose added). The plasma glucose concentration remained higher than the cellular concentration after ten hours of incubation. Yet the amount of glucose in the cells exceeded that in the control cells at the same glucose concentration.

0.001 M IAA, 0.005 M NaF and 0.004 M phloridzin were found to have no effect on utilization or loss from the plasma.

BEEF

*Glucose utilization.* The glucose utilization of three samples of beef erythrocytes was found to be 10.5 mg/100 $cm^3$ cells/hr. when no glucose was added to the blood (fig. 1). Glucose additions were made to aliquots to raise the initial blood glucose concentrations to values of from 165 to 532 mg % (five samples). No difference was found in the utilization of these aliquots, and an aliquot to which no glucose was added (range 9.3 to 12.0 mg/100 $cm^3$ cells/hr.) The rate of utilization remained constant until all of the substrate disappeared, or until termination of the experiment (twenty-four hours).

*Glucose distribution and glucose losses from the plasma.* The glucose present in the whole blood of the animal was entirely recoverable in the plasma as shown in table 6. When glucose was added to the blood, the cells showed no tendency to take up glucose remaining "glucose free" despite prolonged incubation. Twenty-four hour studies with 532 and 2210 mg % glucose present in samples of blood are shown in table 6 (typical of many experiments). Glucose utilization coincided with the glucose losses from the plasma.

*Hexokinase activity of stroma.* Since the glucose losses from the whole blood and plasma were identical, it was of interest to know whether glucose metabolism was initiated at the cell surface. Experiments were performed to determine the hexokinase activity of the cell stroma. Beef hemolysates were prepared by alternately freezing and thawing. The hemolysate was diluted 1 : 3 with a medium containing 0.013 M phosphate buffer (pH 7.4), 0.06 M $MgCl_2$, 0.01 M NaF and glucose. No hexokinase activity could be detected unless Adenosine tri-

phosphate was added. Glucose utilization in whole and stroma-free hemolysates are compared in table 7. There is little, if any, hexokinase activity associated with the stroma of the beef erythrocyte. Glucose utilization in the hemolysates is higher than that of the intact cells. The faster rate of utiliza-

TABLE 6

*Beef: The concentration of glucose in the whole blood and the amount of this glucose recoverable in the plasma and red cells*

| SAMPLE | LENGTH OF INCUBATION HOURS | WHOLE BLOOD MG GLUCOSE/100 CM³ | MG GLUCOSE RECOVERABLE IN THE | |
|---|---|---|---|---|
| | | | Plasma | Cells |
| A | 0 | 86.9 | 85.5 | 1.4 |
| B | 0 | 65.3 | 65.5 | — 0.2 |
| A | 0 | 532 | 534 | — 2 |
| | 12 | 492 | 486 | 6 |
| | 24 | 437 | 434 | 3 |
| B | 0 | 2210 | 2240 | — 30 |
| | 11½ | 2220 | 2250 | — 30 |
| | 24 | 2210 | 2110 | 100 |
| D | 0 | 177 | 176 | 1 |
| | 4 | 153 | 152 | 1 |
| | 8 | 127 | 129 | — 2 |
| | 12¼ | 101 | 99 | 2 |
| | | .001 M IAA also added to the blood | | |
| | 0 | 174 | 170 | 4 |
| | 4 | 174 | 162 | 12 |
| | 8 | 173 | 160 | 13 |
| | 12¼ | 171 | 152 | 19 |
| | 20 | 171 | 153 | 18 |
| E | | .05 M NaF added to the blood | | |
| | 0 | 204 | 199 | 5 |
| | 11 | 191 | 188 | 3 |

tion may be the result of the high concentration of ATP or the removal of the permeability barrier of the cell.

*Effect of inhibitors.* Small quantities of glucose disappeared from the plasma when utilization was inhibited with 0.001 M IAA. The losses as seen in table 6 were smaller than the amount utilized by control cells over the same period.

Although the glucose utilization of these cells was not affected by 0.02 M NaF, a partial inhibition of utilization was found with 0.05 M NaF. The results of the latter case are presented in table 6, and showed the cells to remain "glucose free."

SHEEP

*Glucose utilization.* The glucose utilization of a sample of sheep blood was 19 mg/100 $cm^3$ cells/hr. when no glucose was added. There was little variation in this rate when the glucose concentration was raised as high as 576 mg % (four

TABLE 7

*Beef: Hexokinase activity of whole hemolysates (WH) and stroma free hemolysates (SFH)*

| | MG GLUCOSE/100 $CM^3$ OF SUSPENSION | | |
|---|---|---|---|
| | Initial | 5 hours | Change |
| Medium + ATP (2.25 mg/$cm^3$) | 96.9 | 97.5 | + 0.6 |
| Medium + WH | 72.9 | 76.5 | + 3.6 |
| Medium + WH + ATP (2.25 mg/$cm^3$) | 63.6 | 39.9 | — 23.7 |
| Medium + SFH + ATP (2.25 mg/$cm^3$) | 68.1 | 46.5 | — 21.6 |
| Intact cells in plasma | | | — 12.5 |

samples, range 17.7 to 18.8 mg/100 $cm^3$ cells/hr.). The rate of utilization remained constant until the substrate had disappeared, or until termination of the experiment (19 hours).

*Glucose distribution and glucose losses from the plasma.* Like beef cells, sheep erythrocytes were initially and remained approximately "glucose free" upon addition to the whole blood (vide table 8).

*Effect of inhibitors.* When glucose utilization is inhibited with 0.001 M IAA or 0.03M NaF, small amounts of the 500 mg % glucose added became associated with the cells. The results in table 8 show that these amounts were the same after 19½ hours as they were after 10 hours.

PIG

*Glucose utilization.* The results obtained from a single sample of blood are presented in figure 10 (similar results

were obtained with another sample of blood). The initial blood glucose concentrations were raised to 273 and 597 mg % in two aliquots and no addition was made to a third (197 mg % originally). The glucose utilization, 2.5 mg/100 $cm^3$ cells/hr. did not vary in these concentrations of glucose.

TABLE 8

*Sheep: The concentration of glucose in the whole blood and the amount of this glucose recoverable in the plasma and red cells*

| SAMPLE | LENGTH OF INCUBATION HOURS | WHOLE BLOOD MG GLUCOSE/100 $CM^3$ | MG GLUCOSE RECOVERABLE IN THE | |
|---|---|---|---|---|
| | | | Plasma | Cells |
| A | 0 | 45.0 | 46.2 | — 1.2 |
| B | 0 | 37.1 | 36.2 | 0.9 |
| A | 0 | 197 | 193 | 4 |
| | 4 | 169 | 164 | 5 |
| | 8 | 144 | 141 | 3 |
| | 12 | 116 | 115 | 1 |
| A | 0 | 556 | 556 | 0 |
| | 6 | 500 | 511 | — 11 |
| | 12 | 473 | 463 | 10 |
| | 19½ | 425 | 418 | 7 |
| | .001 M IAA added to an aliquot of blood initially | | | |
| | 0 | 549 | 556 | — 7 |
| | 10 | 554 | 531 | 29 |
| | 19½ | 547 | 525 | 22 |
| | .05 M NaF added to an aliquot of blood initially | | | |
| | 0 | 547 | 545 | 2 |
| | 10 | 547 | 525 | 22 |
| | 19½ | 517 | 498 | 19 |

*Glucose distribution.* The original determinations of fresh pig blood found in table 9 show the cells to be "glucose free." However, some glucose entered these cells on standing, although no glucose had been added to the system. When the bloods of other species investigated were incubated without additional glucose, the cells which were glucose-free initially remained so, and the concentrations in cells which contained glucose originally decreased with time. One explanation for the increases observed with pig cells could be an increased

permeability under *in vitro* conditions. However, if the rate of utilization were lower *in vitro,* while the permeability did not change from an *in vivo* situation, one could expect the same results. The glucose content of the cells rose when glucose was added to the system but remained markedly lower

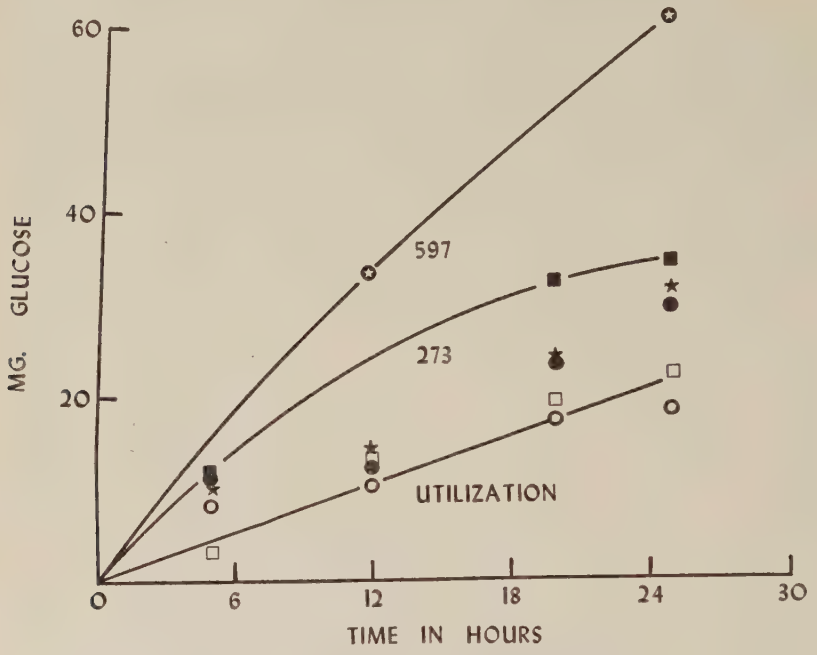



Fig. 10 Pig: The loss of glucose from the plasma and whole blood (utilization) of 100 cm³ whole blood (40% hematocrit initially) after the addition of various amounts of glucose to the blood.

| BLOOD SAMPLE | INITIAL GLUCOSE CONCENTRATION MG/100 CM³ BLOOD | MG GLUCOSE LOSS FROM | |
|---|---|---|---|
| | | Whole blood | Plasma |
| A | 196 | ○ | ● |
| A | 273 | □ | ■ |
| A | 597 | ★ | ✪ |

than the plasma concentrations in the samples described above. These data are also given in table 9.

*Glucose losses from the plasma.* The glucose losses from the plasma increased as the glucose concentration of the plasma was raised. Losses recorded during 25 hours are shown in figure 10.

TABLE 9

*Pig: The concentration of glucose in cells and plasma expressed as mg of glucose/100 cm³ of water in the cells and plasma*

| SAMPLE | LENGTH OF INCUBATION HOURS | MG OF GLUCOSE/100 CM³ OF WATER OF THE | |
|---|---|---|---|
| | | PLASMA | CELLS |
| | No glucose added to the blood | | |
| A | 0 | 389 | 3.4 |
| | 5 | 369 | 13 |
| | 12 | 366 | 13 |
| | 20 | 345 | 23 |
| | 25 | 332 | 42 |
| | 400 mg glucose added to the blood initially | | |
| A | 0 | 1150 | 0 |
| | 12 | 1110 | 46 |
| | 25 | 1070 | 81 |

## HUMAN

*Glucose utilization.* The glucose utilization of human blood was found to be 49 mg/100 cm³ cells/hr. in a single sample of blood (fig. 1). Numerous studies have been made on the permeability of the human red cell for glucose (Mawe, '56; Wilbrandt, Guensberg and Lauener, '47, and LeFevre, '48). However, none of these studies have necessitated the simultaneous measurement of glucose utilization.

## SUMMARY

When the rates of the plasma losses upon addition of 500 mg % are compared, the species can be arranged in this order: rat > rabbit > dog = guinea pig > pig (vide table 10). Tentatively, this might be considered the order of the permeability of the red cells of these species for glucose. (Sheep and beef are omitted here, as they are apparently "impermeable.") This order would also suffice for the rate of glucose utilization at 500 mg % except that dog > guinea pig. If one species had a higher rate of utilization than another, the losses of glucose from the plasma of the former would be larger, even though the two species had the same permeabilities. It is conceivable then that rat, rabbit, guinea pig, dog and pig cells

could be equally permeable, and that the losses from the plasma indicate nothing as to relative permeabilities, but simply reflect the rates of utilization. On the other hand, cells of one species could not both utilize glucose faster and increase in glucose concentration more rapidly than those of a second

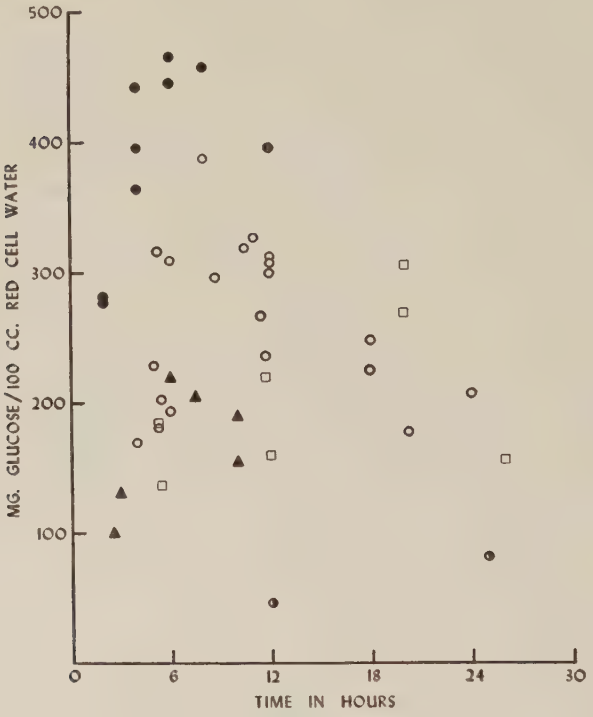



Fig. 11 Cellular glucose concentrations (mg glucose/100 cm³ red cell water) after addition of ca. 500 mg % glucose to: ● = Rat; ○ = Rabbit; ▲ = Guinea pig; □ = Dog; ◑ = Pig bloods.

species unless they were more permeable. The cellular concentrations of glucose after incubation with 500 mg % are shown in figure 11. That the rat cells must be the most permeable follows from their greater utilization rate and more rapid increase in glucose concentration. Using the same criteria, the permeability sequence must be rat > rabbit > dog = guinea pig > pig.

TABLE 10

*The amount of glucose (mg) lost from the plasma of 100 cm³ of various bloods (40% hematocrit) at intervals of time*

| | TWO HOURS | SIX HOURS |
|---|---|---|
| Rat | 120 | 235 |
| Rabbit | 60 | 140 |
| Dog | 40 | 110 |
| Guinea pig | 40 | 110 |
| Sheep | 16 | 43 |
| Beef | 8 | 25 |
| Pig | 5 | 16 |

## DISCUSSION

Two recent reviewers (Rothstein, '54; Danielli, '54) have discussed the problem of glucose movements into various cells and have categorized the probable mechanisms into three groups: simple diffusion, facilitated diffusion and active transport. It is not possible to subject the data presented above to the rigorous mathematical treatment necessary to distinguish among these possibilities. The equation derived by M. H. Jacobs and employed by Mawe ('56) to test for a correlation between the laws of diffusion and glucose permeability of the human erythrocyte has two pre-requisites: first, that the total amount of glucose remain constant in the system and secondly, that erythrocyte volume changes occur solely as a result of glucose entry. Neither of these conditions has been satisfied in these experiments. The amount of glucose utilized during these experiments is considerable because of the long time periods involved. Cell volume changes occurred as a result of changes in pH accompanying lactic acid production (Jacobs and Parpart, '31) and the presence of metabolic inhibitors (Wilbrandt, '40, and observation of the author). Hence, the data must be interpreted in rather "qualitative" terms.

Since the glucose losses from the plasma in beef and sheep bloods are independent of external concentrations, it would appear that Fick's Law of Diffusion is not obeyed, and some mechanism other than that of simple diffusion must operate in the red cells of these species. The metabolism and perme-

ability of glucose in these cells appear to be closely linked, for the losses from the plasma and the whole blood are identical. Furthermore, when the glycolytic system is inhibited by NaF or IAA, plasma glucose losses were very small, one-third or lower (tables 6 and 8) than losses when no inhibitor was present. In sheep cells where 500 mg % glucose was added to the blood, a small amount of glucose left the plasma after 10 hours and no further loss was detectable at 19½ hours (table 8). If a carrier system which was not metabolically linked (facilitated diffusion) were in operation, glucose should have penetrated the membrane and have been recoverable as "free glucose" in the cells, since the cells were no longer capable of metabolizing this compound. On the other hand, it is not likely that glucose metabolism is initiated at the cell surface, as there is no appreciable hexokinase activity associated with the stroma of beef cells (table 7). Perhaps a metabolically controlled carrier system is in operation in these cells. Nevertheless, one cannot dismiss the possibility that the permeability is due to simple diffusion solely, but that the rate of diffusion is so slow that the method used was not adequate to detect it. Thus, in instances of slow permeability other alterations in the cells might mask penetration.

In contrast to beef and sheep cells, the glucose concentrations in the other cells studied and the losses from the plasma increased when these cells were incubated with additional glucose (tables 1, 3, 4, 5 and 9). Other investigators (Masing, '12; Kozawa, '14; Ege, '20) did not find these increases and in the light of the present investigation it is simple to understand why. Masing examined cells after incubation at 37°C for approximately one hour; Kozawa's studies at this temperature lasted fifteen minutes. The order of permeability for these cells found in our studies is of such a magnitude that the amounts of glucose which entered the cells within that time would have been difficult, if not impossible, to detect. Masing did incubate some cells for longer periods of time, but these studies were carried out at room temperature or lower. Ege's investigations were of six hours maximum duration and were

carried out at room temperature. The dependence of the rate of penetration on temperature has been well illustrated by Jacobs, Glassman and Parpart ('35). At lower temperatures glucose movements would be slower than those observed here, and therefore the amounts moving into the cells were probably too small to detect. The order of rate of entrance in the present experiments were shown to be rat > rabbit > dog, guinea pig > pig. Wilbrandt ('38) also measured the permeability to glucose of rat, rabbit and dog erythrocytes and obtained this order: rabbit > rat > dog (dog is listed as having a permeability constant of 0). His determinations of penetrations depended upon the change in osmotic pressure of the cells, hence change in osmotic resistance which results from the penetration of glucose. The reason for the difference in the relative rates of the species in the two investigations is not immediately obvious, but if any factors other than the penetration of glucose were changing the osmotic resistance of the cells, the difference might be explained. Wilbrandt objected to a direct chemical approach for two reasons: the possibility of sugar adsorption on the red cells, and the non-specificity of the chemical analysis for glucose. No evidence for the adsorption of glucose by red cells was found during our investigations. The fact that beef and sheep cells remained "glucose free" when incubated with additional glucose for long periods of time speaks strongly against the adsorption of glucose by at least these red cells. Furthermore, adsorption phenomena are characteristically rapid, and yet the glucose added to the whole blood was completely recoverable in the plasma after a time lapse of five or ten minutes. If adsorption did occur, one would expect some amount of the added glucose to be associated with the cells before the initial determination. In regard to the second criticism, that of the non-specificity of glucose analysis, the method used in this investigation was not available in '38. The $ZnSO_4 - Ba(OH)_2$ method of protein precipitation removes the non-glucose reducing materials which plagued other investigators (Somogyi,

'45). The direct chemical approach, it is believed, is the most satisfactory method at hand for this study.

Wilbrandt ('38) calculated apparent permeability constants for rat, dog and rabbit erythrocytes. He called the constants "scheinbare" because of the utilization of glucose by these cells. However, he used these constants to determine the amount of glucose which would enter the cells under a concentration gradient of 100 mg %. His comparison of the amount which the permeability constant predicted would enter the cells at that concentration gradient with the amount which the cells could metabolize during the same time showed that the amount moved under the concentration gradient was only 1/20 of the amount glycolyzed in the case of rat cells. Wilbrandt's method measures *only* the glucose which is present in the cells at the time of measurement and is only a semiquantitative measurement at best. Glucose which entered the cells and was metabolized cannot be measured by this method, and this amount of glucose was considerable in the present study. In fact, the end product of metabolized glucose would alter the volume of the cells and hence their osmotic resistance by a pH effect (Jacobs and Parpart, '31). Also, the lactic acid in the erythrocytes will increase during incubation and hence the number of osmotic particles other than those of glucose in the cells must be greater during the experimental time course. These difficulties would nullify the values determined by the osmotic resistance method and the permeability constants derived from them. Hence Wilbrandt's statement, "Die Zuckerpermeabilität ist bei Kanichen-, Ratten-, und Hundeblutkörperchen so klein, dass sie die beobachtete Glycolyse nicht deckte" need not be correct.

There is no evidence from our investigations to indicate a direct dependence of the permeability of glucose on its metabolism in rat, rabbit and guinea pig erythrocytes. Glucose losses from the plasma in these bloods are greater than whole blood losses and increase with the external concentration of glucose, while glucose metabolism remains relatively constant. The initial losses of glucose from the plasma are not very

different in the presence or absence of the metabolic inhibitors, IAA or NaF. The plasma glucose losses with inhibitor present are greater than one-half of the corresponding losses without inhibitor, even at 12 hours. The difference can be accounted for presumably by the utilization and consequent lowering of the internal glucose concentration in a cell which is metabolizing this substrate. LeFevre ('48) in his studies with IAA, and Guensberg ('47) with IAA and NaF, reported that these inhibitors had no effect on the glucose permeability of the human erythrocyte. Furthermore, the species differences in the rate of glucose penetration in our experiments remain the same when treated with NaF: rat > rabbit > guinea pig. Morgan et al. ('55) have examined the glucose permeability of rabbit erythrocytes with an experimental design such that they could study both inward and outward movements of glucose. They found that the glucose movements were symmetrical (inward movement equalled outward movement) but could not be accounted for by simple diffusion. This work has appeared in abstract form only. No mention of the glucose concentrations or actual data were given and hence it is not possible to evaluate their results.

Another inhibitor, phloridzin, was shown to diminish the plasma glucose losses in rat and rabbit bloods (figs. 7 and 4) at a concentration of 0.004 M, which did not affect the glucose utilization of these cells. Phloridzin is a well-known inhibitor of glucose movements in the intestine and kidney. The exact mechanism of phloridzin inhibitions is not known, but it has been suggested that the inhibitory action is exerted on oxidations with which phosphorylation of adenylic acid is coupled (Shapiro, '47), on glucose-6-phosphatase (Broh-Kohn and Mirsky, '48), or mutarotase (Kenson, '54). LeFevre ('54) has also suggested that phloridzin complexes with a postulated glucose carrier molecule in the human red cell. Whether the phloridzin inhibition in rat and rabbit cells occurs by one of these mechanisms or in some other manner, one cannot say.

The inhibition is not simply the result of the presence of a large "inert" molecule, for sucrose could not produce the same

effect. However, phloridzin influenced the volumes of the red cells in some way, for the volume changes in the control cells were larger than those of the phloridzin-treated cells (figs. 4 and 7; table 2). Whether these volume influences are related to the glucose movement inhibition observed is not known. However, it must be stressed that since phloridzin-treated red cells failed to make the same volume change as occurred in the control cells; it is possible that this abnormal osmotic behavior reflects a condition within the cell that could alter the apparent permeability of these red cells.

It has been shown in these investigations that beef and sheep erythrocytes might be permeable to glucose only in the physiological sense using Hober's term. The exact mechanism of entrance of glucose into the other cells examined is not thoroughly understood as yet because of the impossibility of distinguishing between the proposed mechanisms. Species differences in glucose permeability hitherto unknown have been established, although the reasons underlying these differences are not known.

## SUMMARY

1. The glucose utilization and the permeability for glucose of the erythrocytes of various mamalian species have been studied at 37°C by chemical measurement of the loss of glucose from whole blood and plasma.

2. Upon addition of ca. 500 mg% glucose, the rate of glucose loss from the plasma showed this relationship: rat > rabbit > dog = guinea pig > sheep > beef > pig. In these systems loss from whole blood is the same except that dog > guinea pig.

3. Beef and sheep erythrocytes were initially "glucose free" and remained so after prolonged incubation (19 to 24 hours) with additional glucose. The amounts of glucose in rat, rabbit, dog, guinea pig, and pig erythrocytes increased as the amounts of glucose in the plasma were increased. The rate at which the cellular glucose concentration increased

upon addition of 500 mg% glucose varied among the species as follows: rat > rabbit > dog, guinea pig > pig.

4. The rates of utilization, losses from the plasma, and increases in cellular glucose concentration are interpreted as indicating that the order of permeability of these erythrocytes is rat > rabbit > dog and guinea pig > pig.

5. Glucose losses from the plasma of rat, rabbit, and guinea pig blood occurred in the presence of concentrations of NaF (and also IAA with rabbit blood) which completely inhibited glucose utilization. These losses are greater than half of those observed in the absence of these inhibitors.

6. Glucose losses from the plasma of beef and sheep blood when the glucose utilization of these cells was inhibited with IAA or NaF were markedly lower (less than a third) than losses in the same bloods in the absence of these inhibitors.

7. Glucose losses from the plasma and volume changes of rat and rabbit cells were smaller in the presence of .004 M phloridzin than in its absence, although the glucose utilization and pH changes in these bloods were not affected by this concentration of phloridzin. The difference in volume change is not solely due to the lack of penetration of glucose, but due in part to the direct influence of phloridzin on cellular volume.

8. Although the results indicated that glucose utilization and entrance into the beef cells were closely linked, hexokinase activity could *not* be demonstrated in the stroma of the beef erythrocyte.

9. The possible mechanisms of the penetration of glucose through the plasma membranes of the erythrocytes used in these investigations are discussed in relation to the present and previous observations.

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## ADDENDUM

Since the submission of this paper further discussion of the preliminary report of Morgan et al. ('55) has been published: Park, C. R., R. L. Post, C. F. Kalman, J. H. Wright, L. H. Johnson and H. E. Morgan. 1956. The transport of glucose and other sugars across cell membranes and the effect of insulin. Ciba Foundation Colloquia on Endocrinology, Little, Brown and Co., Boston, *9*: 240–260.

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